From THE DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
Karolinska Institutet, Stockholm, Sweden

PLURIPOTENT STEM CELL-DERIVED TERATOMA PRESENTS AN EMBRYONIC NEOPLASTIC NICHE FOR *IN VIVO* STUDIES OF NEUROECTODERMAL CHILDHOOD TUMORS

Seema Jamil

Karolinska Institutet

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Cover Photo:
Clinical neuroblastoma tumor growth in PSCT *(for details, see paper IV)*.

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‘O my Lord! Increase me in knowledge.’
al-Quran 20:114

To my family

ABSTRACT

Therapy-resistance and relapse remain problematic in many cancer patients, despite the advancement in anti-cancer therapies. There is a constant need of developing new anti-cancer drugs to combat tumors keeping in mind also the increasing incidence rate of cancers as well as the incidences of relapse. Successful development of new drugs strongly depends on the predictability of the employed preclinical model. Currently used animal models (mostly xenografts and to some extent also gene modified animals) have resulted in progress but still experience some limitations. Many anti-cancer drugs have shown to be working in preclinical models, but proved ineffective in patients. It is therefore very important to develop better pre-clinical models providing and improving clinically relevant predictions should be developed. This study was carried out to test a novel pre-clinical model originally suggested by Tzukerman et al. 2003, using pluripotent stem cell induced teratoma (PSCT). For characterization of this experimental microenvironment, early and late events were studied in PSCT generated from the embryonic stem cell line HS181. Paper I demonstrated the occurrence of a benign embryonic process including increasingly chaotic embryonic tissues. An emerging organoid development was observed exhibiting cellular differentiation with close resemblance to that of the developing human embryo. Presence of also neural areas with prolonged immaturity was frequently observed, and with a morphology similar to that appearing in malignant tumors.

We hypothesized from this that areas of immature neural condensation in PSCT may provide a growth supporting neoplastic niche for neuroectodermal tumors. The findings in paper II and III suggested that the microenvironment in PSCT provide adequate support for growth of neuroectodermal tumors, preferably of childhood origin. To test for clinical relevance of these findings, we next evaluated the PSCT for in situ growth and progression of fresh or frozen/thawed tumor biopsy materials obtained from the surgery of childhood tumor patients at the Karolinska University Hospital. Sections from the PSCT model demonstrated unique *in vivo* capturing of progression and micro invasion of the transplanted patient primary tumors - with striking similarities to the tumor conditions in the young patient. Further, an engraftment tropism was observed for implanted tumor cell lines, as well as for patient tumor specimens. In particular, a Neuroblastoma and a Supratentorial primitive neuroectodermal tumor revealed a clear tropism for engraftment in that the Neuroblastoma exclusively incorporated into looses mesenchyme and the Supratentorial primitive neuroectodermal tumor into condensing neural ectoderm. In conclusion, we have demonstrated that PSCT delivers an *in vivo* environment allowing childhood neuroectodermal tumors to maintain most of their original characteristics from the patient. This suggest that the PSCT environment is especially well suited for the assessment, and a strong complementary pre-clinical model for *in vivo* studies of these tumors.

LIST OF PUBLICATIONS

I. Gertow K*, Cedervall J*, **Jamil S**, Ali R, Imreh MP, Gulyas M, Sandstedt B, Ährlund-Richter L. Early events in xenograft development from the human embryonic stem cell line HS181-Resemblance with an initial multiple epiblast formation. *PLoS ONE 2011;6(11): e27741*

II. Cedervall J, **Jamil S**, Pramsickaite L, Cheng Y, Eskandarpour M, Hansson J, Maelandsmo GM, Ringborg U, Gulyas M, Zhen HS, Kanter L, Ährlund-Richter L. Species-specific *in vivo* engraftment of the human BL melanoma cell line results in an invasive dedifferentiated phenotype not present in xenografts. *Cancer Res 2009; 69(9): 3746-3754*

III. **Jamil S***, Cedervall J*, Hultman I, Ali R, Margaryan NV, Rasmuson A, Johnsen JI, Sveinbjörnsson B, Dalianis T, Kanter L, Orrego A, Strizzi L, Hendrix MJC, Sandstedt B, Kogner P, Ährlund-Richter L. Experimental teratoma from human pluripotent stem cells provides an *in vivo* embryonic neuroectodermal niche for studies on neuroblastoma. *Submitted*

IV. **Jamil S**, Hultman I, Cedervall J, Ali R, Fuchs, G, Gustavsson B, Hendrix M, Skikuniene J, Sandstedt B, Kogner P, Ährlund-Richter L. An experimental neoplastic niche for *in vivo* studies of neuroectodermal childhood tumors. *Manuscript*

* These authors contributed equally

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LIST OF ABBREVIATIONS

ALK	Anaplastic Leukemia Kinase
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CS	Carnegie Stage
CSD	Chronic Sun Damage
EB	Embryoid Body
EC	Embryonal Carcinoma
EG	Embryonic Germ
ESC	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FISH	Fluorescent In Situ Hybridization
GD	Gestational Day
GEM	Genetically Engineered Mice
HE	Hematoxylene & Eosin
hESC	Human Embryonic Stem Cells
hFS	Human Foreskin (cells)
ICC	Immunocytochemistry
ICM	Inner Cell Mass
IHC	Immunohistochemistry
INGRSS	International Neuroblastoma Risk Group Staging System
INPC	International Pathology Classification System
INSS	International Neuroblastoma Staging System
iPSC	Induced Pluripotent Stem Cells
KO-DMEM	Knock-Out Dulbecco's Modified Eagle Medium
KO-SR	Knock-Out Serum Replacement
LOH	Loss of Heterozygosity
mESC	Mouse Embryonic Stem Cells
NB	Neuroblastoma
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PNET	Primitive Neuroectodermal Tumor
PSC	Pluripotent Stem Cell
PSCT	Pluripotent Stem Cell derived-Teratoma
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RTKs	Receptor Tyrosine Kinases
SCID	Severe Combined Immunodeficiency
SNS	Sympathetic Nervous System
sPNET	Supratentorial Primitive Neuroectodermal Tumor
SR	Serum Replacement
SSEA	Stage-Specific Embryonic Antigen
TRA	Tumor Recognition Antigen
TS	Theiler Stage
WHO	World Health Organizatio

1 INTRODUCTION

The research in this thesis includes both experimental and translational angles, where the focus is on studies of neuroectodermal tumors, specifically neuroblastoma (NB), primitive neuro-ectodermal tumor (PNET) and melanoma. Like most other tumors, these tumors are extremely complex, and can be very heterogeneous with regard to their clinical outcome, where some tumors are more benign, while others are more aggressive and have an extremely poor outcome.

Studies of tumors can be done in their natural host, *in vitro* by establishing the tumor cells as cell lines or *in vivo* by growing the tumors in different animal models.
To distinguish these tumors more readily and to be able to later possibly study their response to different therapies, we need better *in vivo* models. Previous models using cell lines *in vitro*, as well as xenografting *in vivo* have had their limitations and to study new models enabling a more differentiated observation of the different cells in a tumor would be of great value.

In this thesis I have studied the use of a novel species-specific *in vivo* model in comparison with traditional xenografts in different ways. However, before diving in more detail into this very specific area a more general introduction of some aspects of this thesis is presented below.

1.1 CANCER

Cancer is a general name (meaning "crab" in Latin and Greek) given to a group of diseases, which are characterized by the uncontrolled cell division, growth and spread and where the spread of the disease destroys its adjacent environment. It is a leading cause of death worldwide. Despite the advancement in the early detection methods and improved therapies, cancer still remains a great challenge for today's health care system. According to a recent report, incidence of cancer worldwide is predicted to increase from 12.7 million cases in 2008 to 22.2 million by 2030 (up to 75%) with a predicted increase in cancer-related deaths up to 13.2 million in 2030 from 7.6 million in 2008 (Bray et al., 2012; Ferlay et al., 2010).

Under normal circumstances, every cell in the body is orderly controlled by the mechanisms that determine when a cell should divide and when it should die. When a group of cells have lost their ability to respond to these homeostasis mechanisms, resulting in an uncontrolled growth of unwanted cells, a cancer may arise. The cells in this cancer may also be capable of invading and metastasizing to tissues far from the organ where the cancer originally started, a characteristic which most of the advanced tumors possess and is a major cause of cancer-related deaths.

Acquisition of tumorigenecity in normal cells is a multi-step process as described e.g. by Hanahan et al (Hanahan and Weinberg, 2000, 2011). During cell division the information coded in the DNA of a cell is transferred to the daughter cells under strict control. Any mutation in the DNA is normally cleared by the DNA repair mechanism

and/or the affected cell is killed by body's immune system. In cases where mutations escape these control mechanisms, these mutations continue to persist/stay in the progeny cells. If these mutations occur in the genes controlling DNA repair machinery, it is expected that the cellular progenies will accumulate multiple mutations due to a lack of efficient repair mechanism. This multi-step acquisition of tumorigenecity may in some cases lead to cancer development.

Cancer cells acquire certain characteristics in order to sustain their growth and to escape different anti-tumor mechanisms – and these characteristics are described as Hallmarks of cancer by Hanahan (Hanahan and Weinberg, 2000, 2011) and illustrated in Fig. 1.

Figure 1: A drawing showing the hallmarks of cancer: Adapted from Hanahan et al, 2010, reproduced with permission from http://www.gettingtoknowcancer.org/overview.php

More specifically Hanahan describes how mechanisms such as, self-sufficiency in growth signals, insensitivity to anti-growth signal, invasion and metastasis to neighboring tissues, limitless replicative potential, sustained angiogenesis and mechanisms to evade apoptosis can be involved in cancer development. Not all mechanisms are involved in all cancers, and in some cancers some mechanisms are more prominent than others.

In this thesis, the focus is on childhood neurectodermal tumors and melanomas and as for many other tumors, in order to design adequate therapies there is a need to understand the nature of these tumors even better. In addition, many of the previous animal models for the studies of these tumors have had their limitation, since they have

not been able to study the interplay between the tumor and its natural environment and the influence the environment has on tumor development and the response to different therapeutic modalities.

The ultimate aim of my thesis is to further elucidate the influence of the adjacent tissues of a tumor on tumor development and hopefully later also on response to therapy. In order to approach this task this thesis has attempted to use a microenvironment potentially more relevant to the studies of neuroepithelial tumors and melanoma, all neuroectodermal. This microenvironment is derived from a teratoma including various human tissues that has developed from human pluripotent stem cell- (hPSC) and more specifically from human embryonic stem cells- (hESC). This project is a continuation of earlier studies done in our group using the potential of hESC-derived teratomas to be a supportive preclinical system. Here I have extended our previous findings on tumors cell lines for use on clinical tumor biopsies with an emphasis on studying childhood cancers of the nervous system.

1.2 NEUROECTODERMAL TUMORS

Neuroectodermal tumors are derived from –neuroectoderm; the part of ectoderm that is to give rise to the embryo's nervous system. These tumors are the focus of my work in this thesis. It concentrates especially on, neuroblastoma, primitive neuroectodermal tumor (PNET), and melanoma. Below is a brief introduction of these tumors and a more detailed description is presented for neuroblastoma and PNET, the tumors dominating in this thesis.

1.2.1 Neuroblastoma (NB)

General introduction

Neuroblastoma is a tumor of sympathetic nervous system (SNS) (Brodeur, 2003). It is the most common extra cranial solid tumor in children and the third most common childhood cancer in general after leukemia and brain tumor. NB accounts for about 7-10% of all childhood cancers and nearly 15% of all cancer fatalities in children. The median age at diagnosis is approximately 18 months and almost 40% of the cases are diagnosed by the age of 1 year (Brodeur, 2003; Maris et al., 2007; Schor, 1999). In Sweden 10-20 children are diagnosed with NB every year (Gustafsson G 2007; Träger, 2009).

NB is assumed to be an embryonic tumor and derived from the derivatives of the neural precursor cells of SNS- the neural crest (Brodeur, 2003; Grimmer and Weiss, 2006; Nakagawara and Ohira, 2004). The neural crest cells in their turn are derived from ectodermal part of the embryo and give rise to a variety of cell types in vertebrates. It is a highly migrative transient population of cells present at the dorsal region of the neural tube closure, beneath the ectoderm. It gives rise to the multipotent progenitors that contribute to the formation of the peripheral nervous system; the enteric nervous system; to melanocytes, to Schwann cells, cells of adrenal medulla; and cells of

craniofacial skeleton. Most NB tumors arise in the abdomen and especially in the adrenal medulla, but they can also occur occasionally at other sites such as neck, chest and pelvis (Maris et al., 2007).

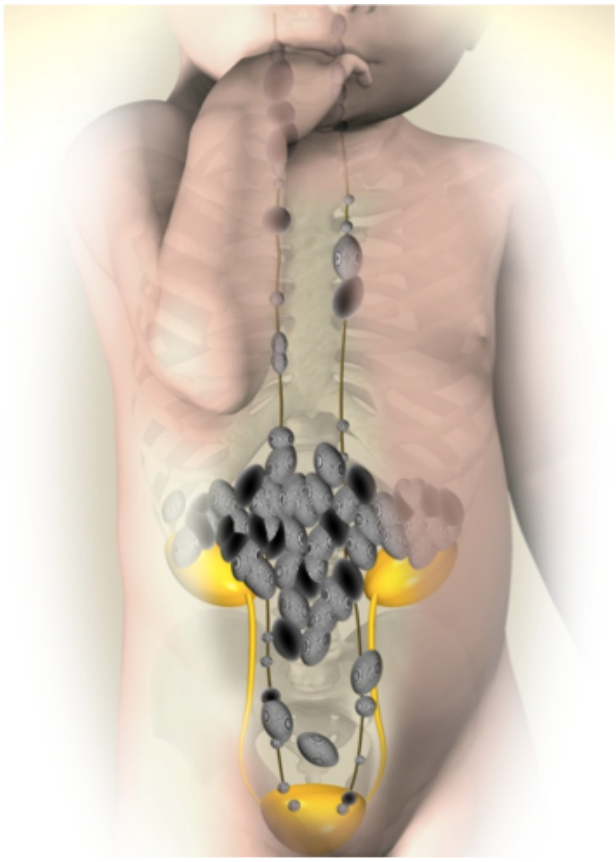

Figure 2: Neuroblastoma primary tumors derived from the neural crest arise in the sympathetic nervous system including the adrenal medulla, sympathetic ganglia and paraganglia. Neuroblastomas mainly metastasize to lymph nodes, bone and bone marrow, and in infants also spread to liver and subcutaneous tissue. Reprinted with permission from Springer Science media and Business media, (Johnsen et al., 2009)

Different classifications

NB is highly heterogeneous both biologically and clinically. The clinical outcome spans from a less aggressive tumor with an ability to spontaneously regress or to differentiate into benign ganglioneuromas, to a very aggressive highly metastatic tumor with resistance to multimodal therapy. According to the International Neuroblastoma Staging System (INSS), NB is classified in to stages 1-4 and 4S (Brodeur et al., 1993). Stage 1 tumors are surgically removed localized tumors often less aggressive without metastases in bone and bone marrow and easily managed without further therapy whereas stage 4, especially in older children, is usually a most aggressive and highly metastatic tumor often resistant to multimodal treatment including chemotherapy and irradiation. Stage 4S is a special stage, in which infants younger than one year with NB have a localized small primary tumor with metastasis in liver, skin or bone marrow and it almost always regresses spontaneously or after minimal therapy (Brodeur, 2003; Maris, 2010; Maris et al., 2007; Schor, 1999; van Noesel and Versteeg, 2004).

NB is also classified into different risk groups according to the risk assessment. A new staging system not taking surgical procedure into account has recently been established by the International Neuroblastoma Risk Group (INRG) as the INRG Staging System (INRGSS) dividing NB into localized and metastatic tumors (L and M or MS, resp) (Monclair et al., 2009). A consensus risk classification is now established with this staging system and other features as age, histology and genetics that divides NB into very low, low, intermediate and high risk groups to be used for clinical management and future studies (Cohn et al., 2009).

Based on histology, NB can be graded as a favorable or an unfavorable tumor. The International Pathology Classification System (INPC) has established a system to grade tumors into good and poor prognosis tumors based on the Shimada histology grading system. This system uses criteria based on degree of differentiation, the Schwannian stromal content and age at diagnosis (Shimada et al., 1999a; Shimada et al., 1999b).

For staging purposes and prognostic assessment, the detection of widespread disease and in particular bone marrow involvement is essential (Brodeur, 1998; Cohn et al., 2009). Recently within the INRG efforts the consensus criteria for detection of neuroblastoma cells in blood and bone marrow were reached (Beiske et al., 2009).

Genetic changes

General: Genetic changes in NB show a close association with tumor stages and aggressiveness and can also be used as prognostic factors. Neuroblastoma presents complex genetics; the acquisition of a number of genetic aberrations including ploidy changes, mutations in certain genes (PHOX2B and ALK), amplification of MYCN, loss of 1p, 11q and gain of 17q (Brodeur, 2003; Maris, 2010). Although none of these genetic alterations has been found as the single causative factor of NB and also none has been found to be generally present in all NB (Bown, 2001; Caren et al., 2010).

Hereditary changes: Hereditary NB accounts for 1-2% NB cases with germline mutations in two genes, ALK and PHOX2B (Fisher and Tweddle, 2012). Anaplastic Leukemia Kinase (ALK) gene was first identified as a predisposition to familial NB and mutations in this gene are now considered to be the most common cause of hereditary NB (Janoueix-Lerosey et al., 2008; Mosse et al., 2008) and familial NB cases are now subjected for the screening of ALK mutations. Besides hereditary NB, mutations in the ALK gene are also present in up to 10% of sporadic NB and in other malignancies as well (Fisher and Tweddle, 2012).

Paired Homeobox 2b gene (PHOX2B) is involved in the normal development of sympathetic nervous system (SNS) and catecholamine synthesis. Mutations in PHOX2B were first identified in Congenital Central Hyperventilation Syndrome (CCHS) and are also present in Hirschsprung's disease, both of them are the diseases caused by the failure of neural crest cell migration and are considered to be associated with a higher risk of developing NB. In such cases presence of mutations in the PHOX2B gene are likely to influence the incidence of NB. Like ALK mutations, PHOX2B mutations are also found to be present in some sporadic neuroblastoma cases (van Limpt et al., 2004).

A brief introduction on other genetic alterations present in NB
Amplification of MYCN: Amplification of MYCN in NB is associated with a poor outcome and it is frequently present in rapidly progressing tumors with advanced stages (Brodeur, 2003; Cohn and Tweddle, 2004; Maris, 2010). In 1983, Schwab et al first described *MYCN* amplification at 2p24 (Schwab et al., 1983). Moreover, MYCN amplification is present in about 20% of all neuroblastomas (Fisher and Tweddle, 2012).

Allelic loss of 1p: Allelic loss of 1p is present in up to 35% NB cases and correlates with MYCN amplification and advanced disease stages (Attiyeh et al., 2005). Many groups have reported 1p deletion as an indicator of worse prognosis.

Gain of 17q: Gain of 17q is the most common genetic aberration in NB and is present in up to 70% of the cases (Caren et al., 2010) and is also related to unfavorable prognosis. It is very unlikely that a tumor is MYCN-amplified or 1p-deleted without a 17q gain (Fisher and Tweddle, 2012).

Imbalance in 11q: 35-40% of NB cases have imbalance of 11q (Attiyeh et al., 2005). Unlike Loss of Heterozygosity (LOH) of 1p, LOH in 11q is not associated with MYCN amplification. Nevertheless, in non-amplified MYCN neuroblastoma, 11q loss is predictive of poor prognosis and characterized by older age at diagnosis and accumulation of a higher number of genetic aberrations (Caren et al., 2010).

Ploidy: DNA index of the tumor cells has been linked to prognosis of NB. Look et al 1984 showed that hyperploidy/triploidy, DNA index around 1.5, in children less than 1 year is linked with early-stage tumors and linked to good prognosis, while diploidy is linked to therapy resistant tumors (Look et al., 1984). Tumors with diploid DNA content have been shown to harbor unfavorable specific genetic aberrations while hyperploid tumors show more favorable pattern of numerical aberrations (Caren et al., 2010).

1.2.2 PNET

PNET is a group of tumors usually occurring in infants, children and young adults. It is assumed to arise from the primitive neuroepithelial cells that are left out from the development of central nervous system (CNS) (Leveine Arnold J., 1993). CNS tumors, in general account for approximately 20% of all cancer cases in children worldwide and are the most leading cause of cancer-related deaths (Heath et al., 2012; Pollack, 2011). In Sweden, 28% of the 300 children with a malignant disease annually have a CNS tumor (Gustafsson G 2007). PNET can be classified into two major types depending on the location of primary tumor, CNS PNET that occurs in the brain and spinal cord and peripheral PNET that is present outside the brain and spinal cord. The most common type of CNS PNET is medulloblastoma that alone accounts for 20% of all childhood brain tumors (Klesse and Bowers, 2010; Samkari et al., 2012). According to WHO classification, CNS PNET also includes malignancies like, supratentorial PNET (sPNET), cerebral neuroblastoma, CNS ganglioneuroblastoma, medulloepothelioma

and ependymyblastoma (Louis et al., 2007; Samkari et al., 2012). CNS PNET, in general present a histopathology composed of poorly differentiated neuroepithelial cells expressing neuronal markers such as synaptophysin (Samkari et al., 2012).

1.2.2.1 sPNET

sPNET are rapidly growing very aggressive tumors that occur in the cerebrum, which is present at the top of the brain and head and makes up the largest part of the brain. The cerebrum controls some of very important functions such as thinking, learning, problem-solving, emotions, speech, reading, writing and voluntary movements (Snell, 2009). sPENT accounts for about 2.5%-3% of all childhood tumors (Jakacki, 1999). Five-year survival rates range from 30%-47% despite maximal therapy (Albright et al., 1995; Cohen et al., 1995; Dirks et al., 1996; Paulino and Melian, 1999; Reddy et al., 2000).

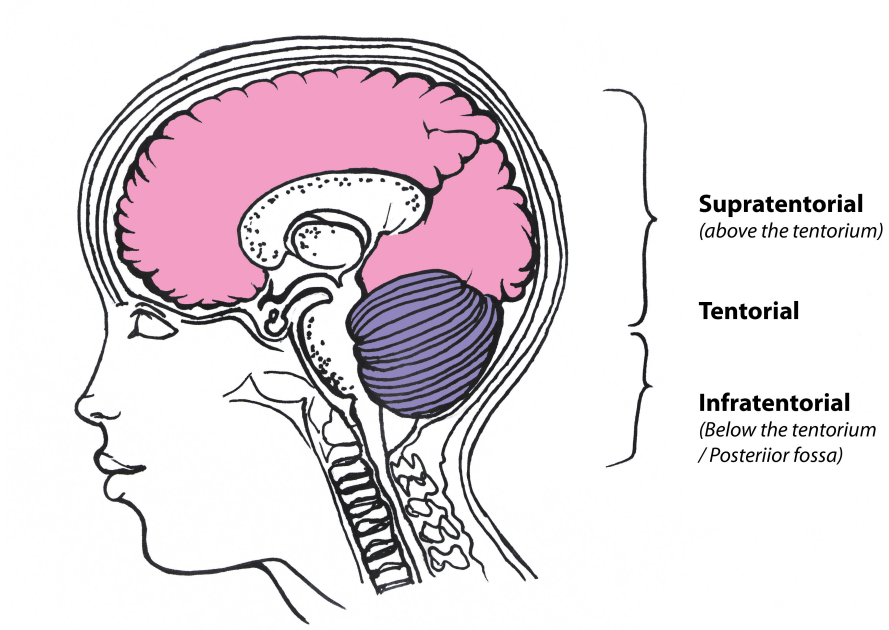

Figure 3: Parts of brain describing the location of supratentorial. Curtsey for drawing, Isabell Hultman.

sPNET are known to have multiple genetic aberrations in contrast to the other CNS PNET such as pineoblastomas and medulloblastomas, which have relatively fewer alterations (Miller et al., 2011). The most common alterations in sPNET are gains of 1q, 2p and 19p and losses of 3p21, 3q26 and 8p23 (Dahlback et al., 2011; Miller et al., 2011).

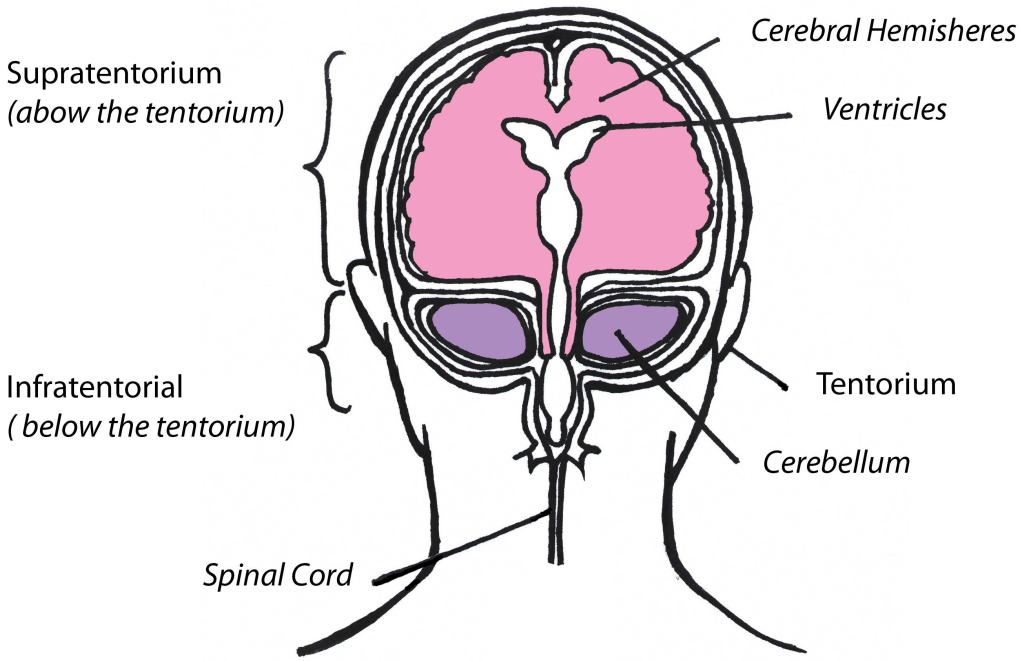

Figure 4: Schematic representation of brain structure. Curtsey for drawing, Isabell Hultman

1.2.3 Melanoma

Melanoma is malignant tumor of melanocytes- the cells that produce a pigment called melanin which gives the skin and hair color. Melanocytes are derived from derivatives of the neural crest of the human embryo. Melanoma can occur anywhere on the skin and is one of the very metastatic tumors. It is the most lethal skin cancers (Jemal et al., 2010). Although malignant melanoma accounts for 4% of all skin cancers, it still causes the highest number of skin-cancer related deaths worldwide, accounting for about 90% of skin cancer mortality (Garbe et al., 2012; Maio, 2012). Melanoma is more common in Caucasians than in other populations and the increasing rate of incidence in the western world is a major health problem (Claeson et al., 2012; Godar, 2011). A recent study carried out by Claeson M and colleagues on the incidence of cutaneous malignant melanoma in the Swedish population during 1970-2007, describes that the number of cases have increased from 557 in 1970 to 2332 in 2007 (Claeson et al., 2012).

Metastasis is the most important predictor of melanoma prognosis (Fernandez-Flores, 2012). Melanoma can metastasize to any organ (Leong et al., 2011). Even small tumors can metastasize, contributing to poor prognosis (Garbe et al., 2012). Resistance to therapy is one of the characteristics of malignant melanoma and hence the biggest challenge to melanoma treatment (Hocker et al., 2008).

Figure 5: A cutaneous malignant melanoma. Reproduced from Wikipedia

Individuals with high numbers of common naevi and those with large congenital naevi, multiple and/or atypical naevi (dysplastic naevi) are at greater risk (Bauer and Garbe, 2003; Garbe et al., 1994a, b; Grobe et al., 1990; Holly et al., 1987). A family history of melanoma occurs in about 10% of melanoma patients and confers an approximately two-fold increase in melanoma risk (Gandini et al., 2005). The most common exogenous factor is UV irradiation (Curtin et al., 2006; Curtin et al., 2005; Tsao et al., 2004).

Melanoma has shown to have dysregulation of signaling pathways for controlling important functions such as cell proliferation, migration and apoptosis. Genetic alteration in BRAF and NRAS genes upstream the MAPK/ERK pathway leads to an activation of this pathway. Mutations in BRAF occur in approximately half of cutaneous melanoma cases (Davies et al., 2002; Hocker and Tsao, 2007). In most of the cases a point mutation leads to an amino acid substitution, which results in the constitutive activation of the MAPK/ERK pathway (Davies et al., 2002; Palmieri et al., 2007). One the other hand mutations in NRAS gene, which is upstream of BRAF, are present in about 15% of all melanoma cases (Edlundh-Rose et al., 2006; Hocker and Tsao, 2007; Omholt et al., 2003; Tsao et al., 2004). These alterations are mainly caused by amino acid substitution in codon 61 (Albino et al., 1989; Ball et al., 1994; Platz et al., 1994). The PI3K-pathway is also observed to be dysregulated in some malignant melanoma cases, and this is mainly caused by an alteration in the tumor suppressor gene PTEN (Bastian, 2003; Wu et al., 2003). PTEN is shown mutated in 60% all melanoma cases (Goel et al., 2006). Dysregulation of MAPK/ERK and PI3K pathways can also be caused due to alterations in one or more upstream receptor tyrosine-kinases (RTKs). Mutations in e.g. receptor tyrosine-kinase *KIT* are present in about 17% of melanomas with chronic sun damage (CSD) (Curtin et al., 2006).

Melanomas are highly plastic tumors and have a great ability to differentiate into multiple cell phenotypes depending on the cues received from the surrounding microenvironment and the need of tumor cell progression. An important example of this plastic potential is the ability of melanoma to participate in vasculogenic mimicry and neovascularization. Melanoma cells are shown capable of differentiating into epithelial cells to support tumor growth and progression. Together with high degree of therapy-resistance, these characteristics emphasize on the need of preclinical models studying factors/cells that support the progression of melanoma and the sustaining of these characteristics.

1.3 EXISTING PRE-CLINICAL TUMOR MODELS

The efficacy of anti-tumor therapy in the patients largely depends on the preclinical model used for testing of the drugs. Hence it is of utmost importance that the model systems used should mimic the natural growth environment of the tumors in patients as much as possible. For obvious reasons human tumors cannot be experimentally grown in humans and *in vitro* experiments and animal models are the available options left.

1.3.1 *In vitro* models – advantages and disadvantages

In vitro studies on tumors have since long been contributing to understanding different cellular and growth mechanisms of cancers as well as for the therapeutic purposes. *In vitro* tumor models are advantageous to study cancer, because researchers have a great degree of control, ease and planning on these experiments. These *in vitro* platforms are cost effective and ethically advantageous over *in vivo* models. Among the scientific advantages, one is that they offer excellent model for studying biological mechanisms of actions due to easier measure of effect in simple, well-controlled experiments and hence *in vitro* models have lead to the discovery of many drugs (Sceats). *In vitro* models can reproduce the properties of tumors to varying extent, including the 3-D growth and matrix interaction (Levenberg et al., 2003; Seftor et al., 2006). However, these models lack the interaction of tumors with the surrounding stroma, and the spatial and temporal coordination of a cellular microenvironment is difficult to attain *in vitro*, which may lead to the drugs being effective *in vitro* but less- or ineffective in the patients (Johnson et al., 2001; Kerbel, 2003; Takimoto, 2001). *In vivo* animal models offer solution to most of the limitations of *in vitro* systems.

1.3.2 *In vivo* animal models

The first *in vivo* models for tumors that were developed in the mid-1960s were mouse leukemia models (Teicher, 2006). There are many inbred, outbred and transgenic species used in laboratories today. Among all the animal models, the species *Mus musculus* is the most extensively studied for transplantation of both mouse and human tumors. There are several reasons to choose mice as models of cancer research: primarily because they are mammals, small in size, easy to handle and maintain. They reproduce quickly and one of the most important reasons is that the whole genome is sequenced and resembles the human genome to a large extent. In addition, its life span of approximately three years offers scientists to design experiments of varying time length.

1.3.3 *In vivo* mouse models- advantages and disadvantages

Experimental mice models have been used as the primary species for the testing of anticancer therapies for over half a century (Kerbel, 2003). Xenografting of tumors in athymic (nude) or severe combined immune deficient mice (SCID) is commonly used.

Xenografts in mice are mostly developed by engrafting the tumor cell lines or primary tumor biopsies either in the original site of tumor growth (orthotopically) or in a location easy to access and monitor e.g. subcutaneously in immunodeficient mouse models. The development of xenograft models for human cancers was a big step closer to achieving more clinically relevant models. The human tumor cell lines can easily be injected. In addition, many of these models can be reproduced, a wide variety of tumor cell lines can be studied, the host animals are readily available, and a large number of animals can be used hence giving a statistically valid data (Teicher, 2006). Nevertheless, despite all the above-discussed advantages, xenograft models still offer a great limitation. A major limitation is that the surrounding environment for tumor growth is non-human. Today it is widely accepted that the influence of surrounding human stroma on the growth and progression of tumors is very crucial specially in testing the anti-cancer therapies. In a patient the cancer is in a context of a complex microenvironment consisting of different cell types including a heterogeneous mass of tumor cells, cancer-associated fibroblasts, endothelial cells contributing to neoangiogenesis and also the host immune system may or may not contribute in the tumor progression. Hence, there is a cross talk between the cells in the cancer and the non-tumor surrounding. This is very important to keep in mind when studying tumor xenografts in mice, and this may have resulted in the inefficacy of many anti-cancerous drugs in clinics. This alteration in the normal tumor architecture of human tumors in mouse xenografts i.e. a disturbed interaction of cancer with its non-tumor environment, could possibly lead to an increased autonomous regulation of tumor growth. Subsequently this could result in a selective pressure from the surrounding leading to a growth of a less heterogeneous tumor mass (De Both et al., 1997; Staroselsky et al., 1992). As a consequence, the therapies developed against these cancers may only be effective against the certain cell types, which are allowed to grow under this selection pressure and may not give the same efficacy against the primary tumors in patients with more heterogeneous tumor masses.

One feature of mice xenograft models of human cancers, especially tumor cell lines, is that the tumors grow rapidly, compared to the slower growth rate in the patient. Hence the progression of tumors in xenograft does not give the real picture of tumor growth progression as it is in patients.

Other more advanced mouse models include genetically modified/Genetically engineered mice (GEM) models harboring and expressing mutated genes. The most important advantage of GEM mice is that the tumors arise ‘de novo’ in the animals and can then be frequently followed over a long period of time. GEMs are good for the studies specially where an intact immune system is required (Kerbel, 2003). However, the still remaining big shortcoming of these models is that the tumors are still murine and not human in nature, and hence the drugs developed against these tumors may not be as effective in the corresponding human situation.

1.4 STEM CELLS

Stem cells are different from other cells in that they can both self-renew to their undifferentiated status, as well as also having the long-term potential to differentiate to more specialized cell types (Thomson and Odorico, 2000). Stem cells are thus present throughout out life, from the fertilized oocyte to adult stage (Fuchs and Segre, 2000; Terskikh et al., 2006).

Stem cells differ on the degree of 'stemness'- i.e. the potential to differentiate into advanced cell types. The highest degree of differentiation potential is totipotency possessed by totipotent stem cells and is retained only by the zygote and up to the eight-cell stage of the morula. As the embryo goes on dividing, the stem cells become limited for their potential to give rise to a complete human body.

1.4.1 Pluripotent stem cell

Pluripotent stem cells are characterized by their capacity of prolonged *in vitro* self-renewal in an undifferentiated state, while maintaining their developmental potential to differentiate into the derivatives of all the three embryonic germ layers both *in vitro* and *in vivo* (Schöler, 2007; Ulloa-Montoya et al., 2005). Embryonic Stem Cells (ESC) are categorized as pluripotent stem cells together with Embryonic Germ (EG) Cell and Embryonal Carcinoma (EC) cells. Martin Evans and Mathew Kaufman derived the first embryonic stem (ES) cells from the inner cell mass (ICM) of mice in 1981 (Evans and Kaufman, 1981). In the same year, Gail R. Martin also derived a pluripotent stem cell line from early mouse embryos (Martin, 1981). The derivation of ES cells from mice lead to the derivation of several other ES cell lines from different species including primates ES cells from Rhesus monkeys (Thomson et al., 1995) and marmoset (Thomson et al., 1996) and eventually to human embryonic stem cells in 1998 (Thomson et al., 1998).

1.4.2 Human embryonic stem cells

In 1998 the derivation of the first human embryonic stem cell (hESC) lines was reported (Thomson et al., 1998). hESC are derived from the ICM of a blastocyst from a pre-implantation human embryo i.e. day 5-6 post fertilization. Cells in the ICM are pluripotent at that stage but loose their pluripotency as the development proceeds, i.e. their pluripotency is a transient condition in the embryo. However if they are extracted and cultured under conditions necessary for the undifferentiated state, they can retain their pluripotency.

1.4.2.1 Culture

To maintain the undifferentiated status of hESC, strict culture conditions are essential. hESC are known to spontaneously differentiate if not kept under optimal culture conditions. Basic Fibroblast Growth Factor (bFGF) helps maintain the pluripotency in

culture and hence is an important component of ES cell culture medium. To avoid biological variation, chemically defined medium can be used and commercially available Serum Replacement (SR) can be used instead of Serum. Human ES cells need an extracellular matrix support for *in vitro* culture. The first human ES cell line was derived on murine fibroblasts as feeder cells (Thomson et al., 1998). However, efforts have been made to avoid animal components as much as possible, and later, several other groups including ours, used human fibroblasts as feeder cells. To avoid the use of feeders at all, feeder-free support systems today are in use, such as Martigel (Klimanskaya et al., 2005; Xu et al., 2001). Feeder based culture is however a robust and cost effective alternative and used throughout this study.

For the quality controls in this thesis work, the hESC were tested for a normal karyotype, expression of nuclear pluripotency markers Oct-4 and Nanog, and cell surface markers Stage-Specific Embryonic Antigen, SSEA-4, Trafalgar; TRA-1-60, TRA-1-81, as well as an absence of markers of differentiation (SSEA-1).

1.4.2.2 Self-renewal and Pluripotency

Self-renewal and pluripotency are the two characteristics that give hESCs a great potential for expansion and differentiation. Self-renewal means continuous cell division while maintaining their undifferentiated state, theoretically giving them a limitless proliferative potential. Pluripotency provides a wide developmental potential to differentiate in to the derivatives from all the three embryonic germ layers, which ultimately give rise to all cells of the human body. Self-renewal and pluripotency are strictly regulated at transcriptional- and cell cycle level. Apart from normal karyotype, human pluripotent stem cell lines express pluripotency cell surface markers Stage-Specific Embryonic Antigen, (SSEA)-3, SSEA-4, Tumor Recognition Antigen (TRA)-1-60, TRA-1-81 and alkaline phosphatase, and absence of markers of differentiation (SSEA-1) of any lineage. (Thomson et al., 1998). On a molecular level, certain characteristics are known to play a role in maintaining pluripotency in hESCs, such as high telomerase activity (Thomson et al., 1998), expression of Oct-4, nanog and sox-2 (Boyer et al., 2005).

Pluripotency of embryonic stem cells can be verified in three ways; by retransferring them into early embryos where they eventually give rise to all somatic cells of the chimeric embryo, including the germ cells. For obvious ethical reasons, such an experiment cannot be performed in humans but has been done in mice (Brinster, 1974; Nagy et al., 1990; Nagy et al., 1993). The other two approaches are to demonstrate that ESC can give rise to the derivatives of all the three germ layers *in vitro* and *in vivo* (Gepstein, 2002).

1.4.2.3 In vitro differentiation

As described earlier that removal of culture conditions maintaining the undifferentiated state of hESC may lead to spontaneous differentiation of hESC. This can easily be seen in non-adherent multicellular aggregates of hESC called Embryoid Bodies (EB). However, to direct *in vitro* differentiation of hESC into a certain cell type, and to demonstrate the pluripotency of hESC in a more controlled manner, various protocols

are in use today hESC into desirable specialized cell types neural lineages (Itskovitz-Eldor et al., 2000; Reubinoff et al., 2001; Schuldiner et al., 2000), hepatic lineage (Touboul et al., 2010), cardiac muscle differentiation (Quattrocelli et al., 2012) etc.

1.4.2.4 In vivo differentiation

For human ESCs, *in vivo* pluripotency is evaluated commonly by induction of benign teratomas upon xenografting in Immune deficient mice- containing tissues representing derivatives of ectoderm, endoderm and mesoderm (Brivanlou et al., 2003; Thomson et al., 1998).

1.5 TERATOMA

As described earlier, teratoma formation is one of the essential criterions for any cell type to be classified as pluripotent stem cell. Benign tumors consisting of several different types of tissues have been described since 17th century (Birch S, 1683). However the term 'teratoma' was first coined in 1863 by Rudolf Virchow (Virchow, 1863).

Teratocarcinomas derived from embryonal carcinoma cells are malignant (Pierce et al., 1960) and thus distinct from the benign teratomas derived from karyotypically normal pluripotent stem cells (hESCs or iPSCs) (reviewed by (Blum and Benvenisty, 2008). Moreover, teratocarcinoma has an abnormal aneuploid karyotype (Oosterhuis and Looijenga, 2005; Ulbright, 2005).

Experimental teratomas are generically equivalent to tumors, but we and others have shown that teratomas derived from pluripotent stem cells with normal karyotypes can also be described as a failed embryonic process without a developmental axis, including increasingly chaotic embryonic tissues with emerging organoid development(Blum and Benvenisty, 2008; Gertow et al., 2004; Lensch and Ince, 2007). This is further emphasized in this thesis (Paper I).

Formation of teratoma has been achieved successfully in various sites in the host animal e.g. subcutaneously, intramuscularly, intratesticularly or under the kidney capsule (Cooke et al., 2006; Gertow et al., 2004; Heins et al., 2004; Plaia et al., 2006). We have chosen, for our studies, intratesticular injections because of several reasons. Of which some are that it is not vital, it is easily located and accessible, immunoprivileged, well encapsulated, keeping the graft in position, and teratoma growth can partly be monitored, by examining it externally. It is likely that the hypoxic and hormonal local environment of testis may influence the growth of implanted hPSC especially at the early stages of teratoma growth when the mouse tissue is dominating the microenvironment. However it should be noted, that at the selected time point i.e. day 45 of teratoma growth for the injection of tumors, the testis tissue is almost completely taken over by the developing PSCT-tissues.

Pluripotent stem cell derived teratomas contain a variety of tissues from ectoderm, endoderm and mesoderm. In 2004, Gertow et al from our group presented a thorough

analysis of the tissues derived from HS181 cell line teratomas (Gertow et al., 2004). The tissues from ectoderm were dominating and moreover neuroepithelium was the most dominant ectodermal derivative. Epithelium of other nature was also detected. Among the mesodermal derivatives, bones and cartilages were detected. Furthermore intestinal and bronchi epithelium of endodermal origin were also detected. These teratomas also presented the embryonic mesenchyme of loose and condensed nature.

1.5.1 Vascularization in teratoma

Several studies have shown that hESC are capable of differentiating into endothelial cells *in vitro* as well as *in vivo* (Gerecht-Nir et al., 2004; Gerecht-Nir et al., 2003; Gertow et al., 2004; Levenberg et al., 2002). Immature human vasculature can be seen but the major contribution towards vascularization is mainly of the host origin. Vessels of human origin can be indicated by positive expression for human CD31 and CD34, and were usually seen at later time points. Gertow et al from our group have reported the appearance of anastomosis of human / mouse vessels, based on chimeric staining for human CD31 (Gertow et al., 2004).

2 AIMS

1. To characterize the embryonic microenvironment in human pluripotent stem cell-derived teratoma (PSCT).

   Paper I

2. To investigate the feasibility of using PSCT for pre-clinical analysis of human tumors, using tumor cell lines.

   Papers II and III

3. To investigate in situ engraftment, growth, and progression of patient tumor material in PSCT.

   Paper IV

3 MATERIALS AND METHODS

Materials and methods are described in the individual papers in more details. Here I have explained in a more sequential manner, step-by-step, collectively for all the papers to give a general idea of the experimental design. Below is a flow chart summarizing the materials and methods of the experimental design for all four articles, illustrated in Figure 6.

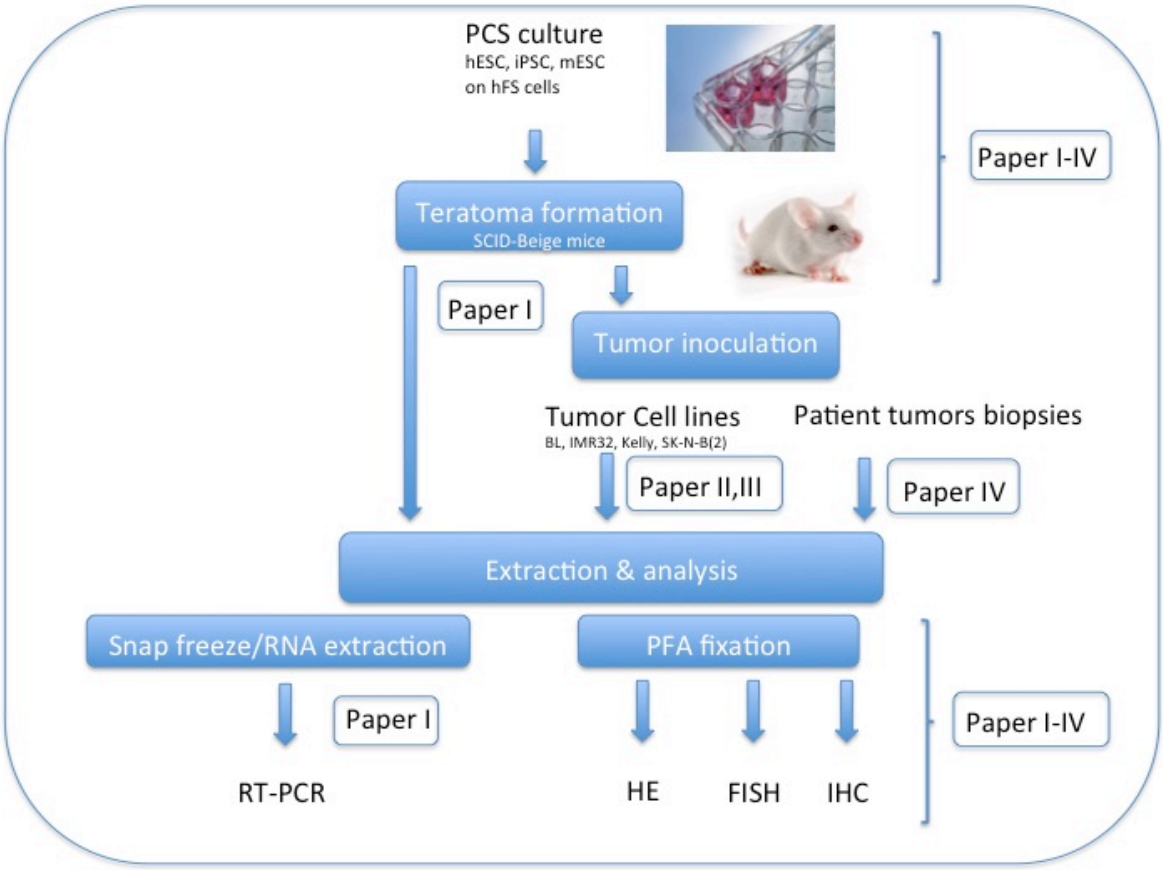

Figure 6: Flow chart of experimental design.

All the studies in this thesis were started with the culture and injections (teratoma formation) of Pluripotent stem cells (PSCs).

3.1 PLURIPOTENT STEM CELLS (PSCS) [PAPER I-IV]

3.1.1 Human Embryonic Stem Cells (hESC)

The human embryonic stem cell lines HS181 (Hovatta et al., 2003) and H9 (Thomson et al., 1998) are the two cell lines that are used to carry out experiments in paper I-IV and paper II, III respectively. Both cell lines were derived from the inner cell mass of human blastocyst and have normal female genotype i.e. 46[XX].

3.1.2 Culture conditions

HS181 and H9 were cultured on human foreskin fibroblast cells (hFS) (CRL-2429; ATCC) as feeder cells. hFS were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen). hFS were mitotically inactivated either by irradiation with 35 Gy before seeding [paper I] or by treatment with mitomycin C (10 μg/ml, 3hours) [paper II-IV]. The fibroblasts were then seeded at 2x104 cells/cm2 on 6-well plates (BD Falcon).

hES were cultured in Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) containing 20% Knock-Out Serum Replacement (KO-SR), 2 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 4ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen). Cells were cultured at 37°C, and 6.8% CO_2 and high humidity (95%). Culture medium was changed daily. The cells were passaged using Dispase (10mg/ml) (Invitrogen) for 5-7 min at 37°C with mild mechanical separation.

In paper III, two other pluripotent stem cell lines were used to generate teratomas. Following are the details about these lines.

3.1.3 Human induced Pluripotent Stem Cells (hiPSC)

hiPSCs were kindly provided by Dr Ludovic Vallier , University of Cambridge, UK (Vallier et al., 2009). These cells were derived from adult dermal fibroblast and were grown in Advanced DMEM/F-12 (Invitrogen) supplemented with 20% (v/v) Knockout Serum Replacement (Invitrogen), 2mM L-glutamine (Invitrogen), 0.1mM B-mercaptoethanol (Sigma) and 4ng/ml basic Fibroblast Growth Factor (bFGF, R&D).

3.1.4 Mouse Embryonic Stem Cells (mESC)

mESC RW-4 were kindly provided by Dr Stephan Teglund, Karolinska Institute and were originally derived from inner cell mass of mouse blastocyst. mESC were cultured on murine embryonic fibroblasts (MEFs) as feeders cells in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 15% FBS (Göteborgs Termometerfabrik).

3.1.5 Quality control

Mycoplasma: All experiments were performed on Mycoplasma free cell cultures. Mycoplasma infections were screened using EZ-PCR Mycoplasma Test Kit (Biological Industries). Cell were cultured for 3 days without changing medium and supernatant was tested for the presence of Mycoplasma using the PCR reaction mix, according to the manufacturer's recommendations. The experiments were performed together with a positive control provided with the kit.

3.1.6 Pluripotency

RT-PCR: In vitro pluripotency of hESC was routinely characterized by RT-PCR for markers of pluripotency. Two different RNA extraction methods were used for this purpose, RNA was extracted using Micro-to-Midi kit (Invitrogen) from *in vitro* cultured cells or EBs [Paper I] and TRIzol extraction was used in paper II-IV. Preparation of cDNA and PCR methods was same as described in sections 3.5.4 and 3.5.5.
See Table 2 for details on Primers (Cybergene) and conditions used for characterization of hESC.

Table 1: RT-PCR; primer sequences and conditions used for characterization of hESC.

Gene	Primer sequence	Size (bp)	Annealing Temp (°C)	mM $MgCl_2$
β - actin	F:5′-GACATTAAGGAGAAGCTGTGCTATGTT-3′ R:5′-GCCTAGAAGCATTTGCGGTGGACGA-3	497	58	3.0
GAPDH	F:5′-GCTCAGACACCATGGGGAAGGT-3′ R:5′-GTGGTGCAGGAGGCATTGCTGA-3	470	55	3.0
Nanog	F:5′-CGGCTTCCTCCTCTTCCTCTATAC-3′ R:5′-ATCGATTTCACTCATCTTCACACGTC-3	960	57	1.5
Oct4	F:5′-AGGATCACCCTGGGATATACACA-3′ R:5′-AAGCTAAGCTGCAGAGCCTCA-3′	113	55	3.0
hTERT	F:5′-CGGAAGAGTGTCTGGAGCAA-3′ R:5′-GGATGAAGCGGAGTCTGGA-3′	147	51	1.0
Klf4	F:5′-CTGCGGCAAAACCTACACAA-3′ R:5′-GGTCGCATTTTTGGCACTG-3′	182	51	3.0

ICC: hESC were characterized for markers of pluripotency by performing Immunocytochemistry (ICC) in papers II-IV. For this, cells were fixed in 4% PFA, permeabilized using 0.25% Triton X. Unspecific binding was blocked using 3% Bovine Serum Albumin (BSA). Primary antibodies were applied for either 1 hour at room temperature or overnight at 4°C, followed by application of secondary antibody for 30 min. Cells were counterstained with DAPI and Zeiss Table 3 shows the details of these experiments.

Table 2: Antibodies used for hESC characterization.

Antibody	Dilution	Source
Oct4	1:50	Chemicon, MAB4401
Nanog	1:50	R&D, AF1997
TRA1-81	1:50	Chemicon, ab90233
TRA1-60	1:50	Chemicon, ab90232
SSEA-4	1:50	Chemicon, ab90230

3.2 PSCT-DERIVED TERATOMA IN SCID-BEIGE MICE: [PAPER I-IV]

3.2.1 Animals

Animal experiments were carried out on 6-8 weeks old male Severe Combined Immunodeficiency (SCID)-beige (C.B.-17/GbmsTac-scid-bgDF N7) mice, obtained from M&B, Denmark. SCID-beige mice carry two mutations; SCID mutation that results in lack of B and T lymphocytes and Beige mutation that causes impairment of NK-cell function and macrophage defects (Croy and Chapeau, 1990; MacDougall et al., 1990).

Animals were kept at the MTC animal facility using the following conditions: temperature at 20°C to 24°C, 50% relative humidity, 14 /10 hour light-dark cycle. The animals were kept with food and water ad libitum.

How it was done

PSC-derived teratoma formation was performed by the injection of 1x104 -1x105 PSCs in 20µl culture medium under the testis capsules of 6-8 weeks old SCID-Beige mice under anaesthesia (3.0 % isofluran).

PSC were mechanically cut and collected in a 1-ml syringe with 27G¾´´ Nr. 20 0.4-19mm grey needle (both from BD Falcon) immediately prior to injection. The cells were allowed to engraft for 6-8 weeks resulting in a benign teratoma containing the tissues from all three germ layers.

In paper I, the experiments were terminated at this stage and the teratomas were extracted at indicated times points for analysis (Sections 3.4 & 3.5). In Paper II-IV, either tumor cells or a piece of tumor from surgery was inoculated into 45-60 days old teratomas. Details are as follows.

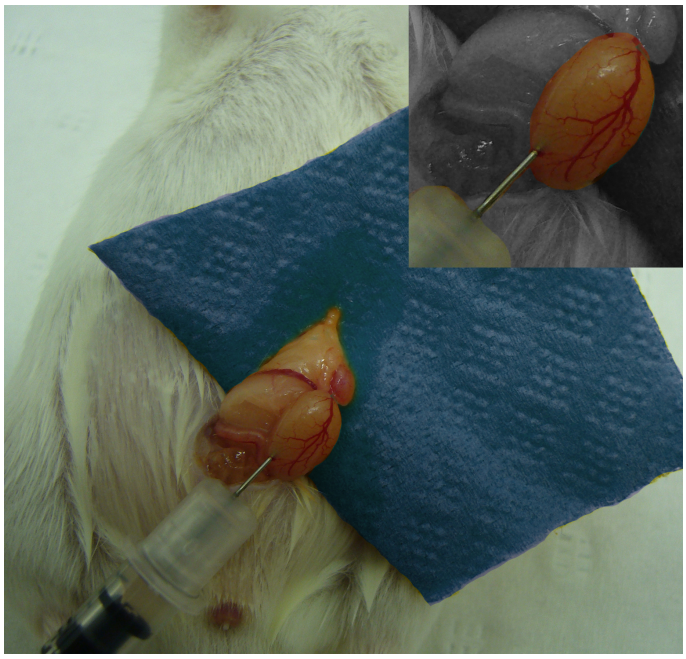

Figure 7: Intratestis injection of pluripotent stem cells to induce PSCT.

3.3 TUMOR INOCULATIONS: [PAPER II-IV]

3.3.1 Tumor cell lines

BL-Melanoma cell line: [Paper II] was previously derived from a lymph node metastasis of a malignant melanoma from a male patient treated at Karolinska University Hospital (Salazar-Onfray et al., 2002).

Neuroblastoma (NB) cell lines: [Paper III] Three Neuroblastoma cell lines IMR-32, Kelly and SK-N-BE (2) were used. NB cell lines were obtained from ATCC.

IMR-32: was derived from the abdominal tumor mass of a 13-months old Caucasian male neuroblastoma patient. The tumor line has an amplification of MYCN gene, together with partial chromosome 1 deletion (Tumilowicz et al., 1970).

Kelly: was derived from a brain metastasis of neuroblastoma. It has MYCN amplification, gain of chromosome 17q, and deletion of 11q as the major aberration.

SK-N-BE (2): was derived from the bone marrow metastasis of a disseminated neuroblastoma after repeated cycles of chemotherapy and radiotherapy from a two year old child in 1972. The tumor has an amplification of MYCN and a deletion in tumor suppressor gene p53.

All tumor cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen).
All tumor cell lines were tested for mycoplasma absence by PCR discussed above (section 3.1.5).

3.3.2 Clinical tumors

In Paper IV, fresh or frozen clinical tumor biopsies from surgery were injected/inoculated into the pluripotent stem cell derived-teratoma (PST) or as xenografts. Tumor specimens were received from pediatric patients at the Karolinska University Hospital for the studies.

Selection criteria for tumors included neuroectodermal origin with male gender: i.e. stable Y-chromosome or presence of a tumor genetic marker e.g. amplification of MYCN gene to enable the distinction of tumor cells from the PSCT-cells (originated from the female; 46XX HS181 cell line). Total nine patients were recruited for the study, out of which three female patients without amplified MYCN were not used in experiments for technical limitations. Fresh tumor material was collected at surgery and transported to the laboratory as fast as possible (15 minutes). In the laboratory the material was divided into aliquots using a scalpel. One aliquot was injected into the PSCT *in vivo* model as fast as possible (within one hour). Cryopreservation of aliquots; DMSO were saved in liquid nitrogen to be thawed at chosen time points.
Table 1 below, briefly describes the diagnosis and clinical history of the six patient tumor samples used in the study, for more details please see, Table 1 in Paper IV.

Table 3: Details of surgical tumors used in Paper IV.

	Diagnosis	Stage	Type of biopsy	Chr. Aberration
Patient 1	Supratentorial Peripheral Neuroectodermal Tumor (sPNET)	2nd relapse very aggressive irradiated and chemo	Surgical biopsy, pieces of tumor	Not done
Patient 2	PNET-like tumor	Undifferentiated infratentorial tumor with sarcomatous features of supposed neuroectodermal origin	Primary tumor	Molecular analysis did not find any evidence of translocation
Patient 3	Pilocytic astrocytoma of the brainstem	low malignancy	Primary tumor Hydrocephalous	N/A
Patient 4	Classic Medulloblastoma	highly malignant	Primary tumor	Not done
Patient 5	Esthesio Neuroblastoma	Non--irradiated	Primary tumor	EwS-Fli1 translocation
Patient 6	Neuroblastoma	M (INRG), 4 (INSS)	Metastasis in skull, first relapse	11q-

3.3.3 Tumor inoculation in xenograft and PST

How it was done

Cell lines: Adherent tumor cells were enzymatically detached, counted, and suspended in ice-cold PBS (kept on wet ice), and 20µl PBS containing 1-2x10^6 viable tumor cells injected either directly under the testis capsules of anesthetized SCID-Beige mice as xenograft or into the PST. Tumors were allowed to engraft for two weeks before the material was extracted.

In Paper II, xenografts of BL melanoma cell lines were also made subcutaneously. (See Paper II for details).

Clinical tumors: Tumors were either inserted as a small piece (1mm^3) or injected as cell suspension. In most of the PSCT cases and in some xenografts, the tumor was inserted as a small piece by either making a cut by a sharp surgical disposable scalpel (BRAUN, Aesculap Division) or making a hole by a 20G 1½´´ Nr. 1 0.9-40mm yellow needle tip (BD Falcon) in the testis and/or PSCT. In case of PSCT, due to the fluid pressure from inside, it was extremely hard to make the tumor piece stay inside the teratoma, that's why a suture was made on the teratomas on most of the cases using Vicryl 199mm ⅜ c sutures (Ethicon). For more details on individual samples Please see Table 2 in Paper IV.

Cell suspension was made by plunging with the plunger of a 5-ml syringe (BD Falcon) and filtering the cell suspension through 70µm Nylon cell strainer (BD Falcon) The suspension was then injected as xenograft or in PST using a 1ml-syringe (BD Falcon) and 27G¾´´Nr. 20 0.4-19mm grey needle (BD Falcon).

3.4 EXTRACTION: [PAPER I-IV]

The extraction method was the same in papers I-IV, but differed regarding the time point for extraction. In paper I, PSCT were extracted at indicated time points (Day 5, 10, 20, 30, 45 and 60). In papers II and III, PSCT/tumors containing tumor cell lines and tumor xenografts were extracted two weeks after the injection of tumors cells.

In paper IV, the shortest observation period was 18 days and the longest period of growth of clinical tumors was 90 days. The upper limit was determined by the humane endpoint from the host animal point of view, i.e. before the PSCT/tumor reached a total size of 15 mm diameter (allowed according to the ethical permit).

How it was done

After the end point of experiment was reached, the animals were sacrificed by cervical dislocation and teratoma [Paper I]/teratoma+tumor and tumor xenografts [Paper II-IV] were harvested.

In papers I and IV, the material was divided in two halves with a sharp blade for either histology or cryopreservation. In Paper II and III, all the tissues were fixed in 4% Paraformaldehyde (PFA) since only histological analysis was done and no RNA was extracted.

3.4.1 PFA-fixation

One half of harvested material was fixed in 4% PFA overnight at 4°C. The tissues were dehydrated through a series of alcohol to xylene, embedded in paraffin and serially sectioned into 5µm thick sections for analysis. Non-injected testes were also fixed in the same manner to use as negative control for FISH experiments [Paper I].

3.4.2 Snap freezing

The other half of the extracted material was snap frozen in liquid nitrogen and saved in -80C until used. In paper I, frozen material was used to extract RNA. In paper IV, frozen material was saved to enable further analysis in future.

3.5 ANALYSIS: [PAPER I-IV]

PFA-fixed tissues were used for the following techniques:

3.5.1 Histology: Hematoxylin & Eosin (H&E)

Standard H & E was performed on 5µm thick sections to detect the engraftment of hESC [paper I] and to study the histology of engrafted tumors [paper II-IV].

Histology, and details of tumor cell differentiation was performed accordingly to routines used in daily clinical practice and as described (Joshi et al., 1992; Shimada et al., 2001) and in a previous study (Gertow et al., 2007).

This part of the project was performed in collaboration with experienced pathologists, Bengt Sandstedt (Karolinska Institutet; department of women's and children's health and Danderyds Hospital), Jurate Skikuniene (Karolinska Institutet, department of Onclology and Pathology Karolinska University Hospital), and Luigi Strizzi, (Children's Hospital of Chicago Research Center, Cancer Biology and Epigenomics Program, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA).

3.5.2 Fluorescent In Situ Hybridization (FISH)

A human specific FISH probe (Spectrum Red labeled total human genomic DNA, Vysis Inc) was used to verify the engraftment of hESC in the testis and also for the human origin of the formed tissues. [Paper I]. In paper II-IV, engraftment of XY tumors with stable Y chromosome was detected by the X and Y-chromosome specific

FISH probe (CEP X SpectrumOrange CEP Y SpectrumGreen, Vysis Inc.), and the tumors without a stable Y-chromosome and female tumors having MYCN amplification were detected by a FISH probe LSI n-myc, 2p24 (Vysis Inc.).
Xenograft sections from the tumor cell lines were used as positive control, and xenografts from tumors lacking a Y-chromosome or NMYC amplification were used as negative control.

The recommended protocol by manufacturer was same for all the probes except for variable hybridization temperature for different probes. Briefly, the sections were deparaffinized using xylene, rehydrated using a series of Ethanol and water. Sections were then pretreated by boiling in Citrate buffer (pH 6.0) and pepsin at 37°C for 5 min. At 75°C for 5 min, double-stranded DNA and probe was denatured, probe hybridization was carried out at recommended temperatures (either 37°C or 42°C) overnight. Sections were fixed with Vectasheild containing DAPI.

FISH to verify the presence of Y-chromosome or NMYC amplification, was used also on tumor cells in culture. For this purpose, cells were grown on gelatine-coated glass chamber slides, fixed by ice-cold methanol for 5 min, and immediately treated for denaturation and hybridization with the same procedure as described for tissue sections.

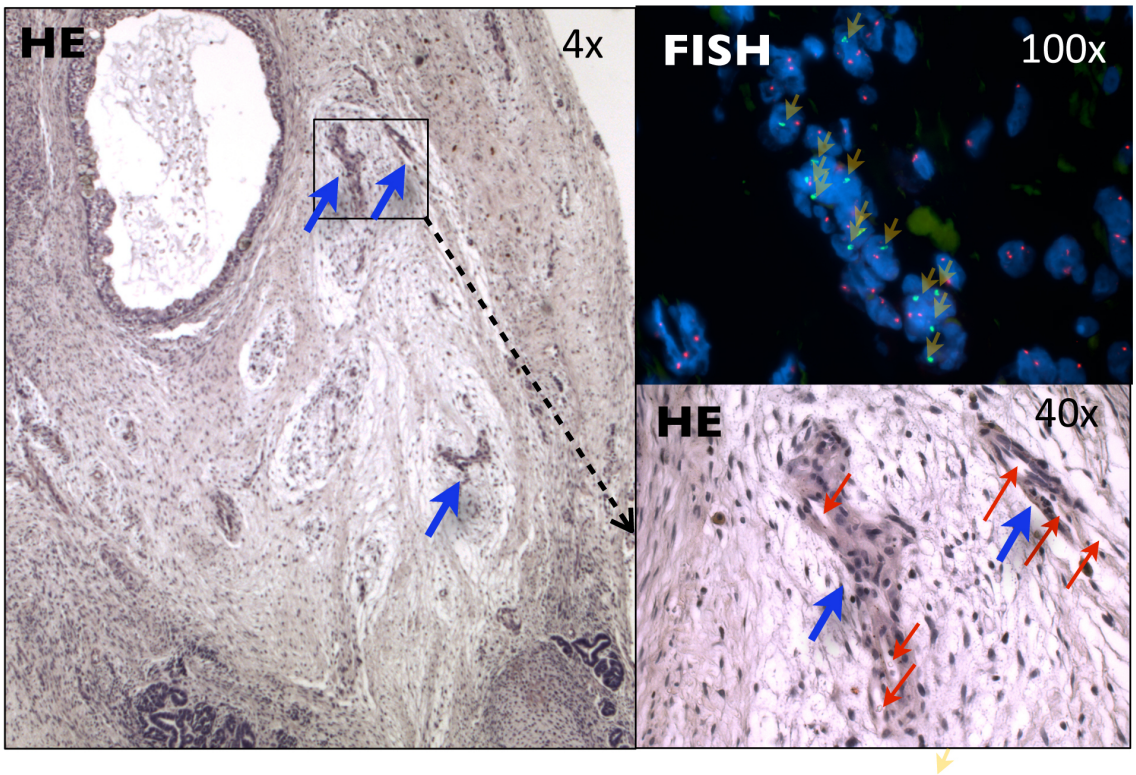

Figure 8: Growth of clinical neuroblastoma in PCST. To the left, NB is growing in areas of loose mesenchyme (blue arrows). Upper right photo illustrates the positive detection of tumor cells (yellow arrows) using a XY chromosome specific FISH probe. Lower right photo shows the tumor growth (blue arrows) close to the blood vessels (red arrows). (X=red, Y=green)

3.5.3 Immunohistochemistry (IHC)

IHC is extensively used in all papers to study protein expression of tumors, and in paper I to analyze the protein profile of different stages of teratoma formation, using a broad panel of antibodies. Paraffin-embedded PFA-fixed sections were used for these experiments. Sections were pretreated according to the optimization protocol of each antibody used. It was either boiling in low pH buffer (for example Citrate buffer), or high pH buffer (like Tris-EDTA buffer), or in some cases no pretreatment was needed. (See individual papers for details regarding conditions and secondary detection system used [Paper I-IV]).

Sections known to express the protein of interest were used as positive control and tissues known to be negative for markers and use of unspecific isotype antibodies were employed for negative control experiments.

Snap-frozen tissues were used for RNA extraction

3.5.4 RNA extraction

In paper I total RNA from half teratomas was extracted using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Extracted RNA was DNAase-treated using DNase 1 amplification grade (Invitrogen) to avoid contamination of genomic DNA. Complementary DNA (cDNA) was synthesized from 20ng RNA, using Superscript III First Strand Synthesis System (Invitrogen) according to the recommended protocol.

3.5.5 RT-PCR

RT-PCR was done on the cDNA obtained from total RNA (See above), using Platinum Taq DNA polymerase (Invitrogen).
See Table S3 in Paper I for Primers used in Study I.

3.6 ETHICAL CONSIDERATIONS

This study was performed in strict accordance with permission for experiments using human embryonic stem cells, from the Local Ethics Committee at Karolinska Institute (114/00), and for animal experimentation from the regional ethical committee (Stockholms norra djurförsöksetiska nämnd; Dnr S172-03 and N105/07). Collection of fresh clinical material from primary surgery at the Karolinska University Hospital was performed with permission number 2008/307-31.

3.7 IMAGE ANALYSIS

Image analysis was done using a Zeiss Axiovert 200M microscope and Q-Imaging software.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Experimental teratoma forming the basis of the PSCT model

Aims of the study

One way of verifying pluripotency of human stem cells is to produce experimental teratomas, representing tissues from all three germ layers, upon xenografting into immunodeficient mice (Brivanlou et al., 2003; Thomson et al., 1998). Ectopic implantation of human pluripotent stem cells in mice results in differentiation defined as the formation of experimental teratoma, and karyotypically normal stem cells induce benign development.

These teratomas are generically referred to as a tumor, but it has previously been reported that benign pluripotent stem cell induced teratomas (=**PSCT**), can be described as a failed embryonic process including increasingly chaotic embryonic tissues within emerging organoid development (Blum and Benvenisty, 2008; Gertow et al., 2004; Lensch and Ince, 2007). Furthermore, despite lacking a developmental axis, such components can show striking similarities to the events in the human embryo.

The teratoma assay has been used to validate *in vivo* pluripotency of stem cells since the 1950th (Stevens and Little, 1954) but still little is known about the most early developmental processes *in vivo* leading to the formation of mature experimental teratomas. Here, we reported for the first time a kinetic analysis of events taking place during the HS181 development *in vivo*, starting from the very early time point, day 5 post injection, with a gradual progression in to mature teratoma until day 60. The study also attempted to align the events in the development of hESC-derived teratoma with that of normal human embryos; an effort to find similarities to normal human development.

The main findings of this study were as follows:

Early events of HS181 development resemble initial multiple epiblast formation

A well-characterized human embryonic stem cell line, HS181, was injected under the testis capsule of the immunodeficient SCID-beige mice for kinetic studies on teratoma formation. Engraftment of/teratoma formation from HS181 cells was analyzed at specified time points, day 5,10, 20, 30, 45 and 60 post injection. The human origin of the engrafted cells was determined by human specific FISH probes and the growth was then analyzed using a large panel of antibodies and RT-PCR markers. Engraftment and active proliferation of hESC progeny was detected already at the first observation time point, day 5. Multiple sites of engraftment were characterized by the presence of single or pseudostratified columnar epithelium, surrounding small cavities, resembling embryonic ectoderm also supported by histological analysis. The neural nature of

growing ectoderm was supported by the expression of early neural marker NESTIN in all epithelia in this stage.

Expression of markers such as Oct4, Nanog, SSEA-4 and KLF-4 supported the formation of epiblast-like structures. The histology and marker analysis of the primitive ectoderm at this stage was well aligned with Theiler Stage (TS) 8 in mouse and Carnegie Stage (CS) 5 (gestational day 7-12) (Larsen, 2001) in human embryonic development. Understanding of the early development of HS181 cells is important with respect to finding out what these cells actually represent *in vivo*, i.e. what natural counterparts they have. Cells in ICM do not sustain prolonged self-renewal and hESC can be regarded as an *in vitro* artifact. Our findings suggest that "growth" from HS181 cell at this stage resembled epiblast formation in embryonic development.

Advanced structures appeared following development at day 30

By day 30, teratomas developed into diverse tissue types, causing almost complete destruction of the mouse testicular parenchyma. Expanding multi-layered epithelium still maintained dominance in tissue distribution. However, other structures were also observed, e.g. a structure with characteristic features of early limb was seen in one exceptional case. This showed a centrally condensing mesenchyme positive for CD56, (marker for limb morphogenesis and early pre-cartilaginous development) covered by thin two layered P63 and E-cadherin positive epithelium with peripheral layer also expressing CK18, an IHC profile similar to that of early epidermal development.

Organized and complex structures appeared at day 60

In mature teratomas (days 45-60), more complex structures representing tissues originating from all three germ layers were found, although in a disorganized manner and lacking a developmental axis. Neuroectodermal structures were still predominant but also tissues of non-neural nature were observed, such as early gut and bronchi-epithelium, renal structures representing presence of intermediate mesoderm, and cartilage of mesodermal or eventually ectodermal neural crest origin. Although a restricted *in vivo* potential for development of HS181 derived tissues was indicated by the absence of mesoderm structures compatible with somites and also lack of structures suggesting early heart formation throughout the study. Somites are derived from mesoderm development and give rise to vertebrae, rib cartilage, musculature and skin of the back, the ribs and the limbs, tendons and some blood vessels but also specify the migration paths of other parts of embryo such as migration of neural crest cells (Bronner-Fraser and Stern, 1991; Keynes and Stern, 1984; Teillet et al., 1987). Absence of somites may have played a role in the chaotic distribution of tissues. Neural dominance was apparent at all stages of teratoma formation, which could also be a possible explanation of restricted development of hESC progeny. One possible explanation of the strong bias for neural development could be a predisposition for the neuroectodermal lineage already in the cultured HS181 cells i.e. before injection.

A rare finding at day 60 was the detection of advanced structures compatible with renal development, found in association with the condensed mesenchyme of nephrogenic

character, and positive for WT1 staining, and with partially vascularized primitive glomeruli and tubuli.

HS181 teratomas were even at day 60 well encapsulated and expanding rather than infiltrating the testicular capsule, with no evidence of malignancy.

A unique approach used in this study was to compare morphologies of different structures in the HS 181 derived teratoma development with that of original human development in the embryos at diagnosed gestational ages. This comparison revealed minor differences in the timeline and histology of developing structures.

Three notable observations support this notion. One was the above-mentioned development of a limb bud. In human development upper extremities appear from CS12 corresponding to gestation day (GD) 28 followed by the emergence of lower extremities at CS14 corresponding to GS 33-35. The observation in PSCT at day 30 (i.e. ICM culture/differentiation for 6 days – *in vitro* expansion with impeded differentiation – and *in vivo* differentiation for 30 days in PSCT) indicated an approximate agreement with the developmental timeline of GS 28-35 for human embryos to develop upper / lower limb buds.

Two other observations at day 53 of a single case of trachea-like embryonic structure with suggestive respiratory epithelium as well as a most advanced structure of a single neural tube like structure were both similarly time matched with only a one week discrepancy to reference material from a human embryo approximately CS21-22 corresponding to GD 53-56.

Several studies have shown that hESC are capable of differentiating into endothelial cells *in vitro*, but showing only immature vessel formation *in vivo* (Gerecht-Nir et al., 2004; Gerecht-Nir et al., 2003; Gertow et al., 2004; Levenberg et al., 2002). In HS181 teratoma development, vascularization was mainly of the host origin although immature vessels of human origin, positive for human CD31 and CD34, were also seen at later time points. Appearance of partly chimeric vessel structures was observed, indicating anastomosis of mouse and human vessels.

4.2 PAPERS II-IV

The need for better pre-clinical models and the use of the PSCT model

In pre-clinical drug development, accurate prediction of efficacy in patients is essential. For the field of oncology however, the attrition rate in clinical trials is unacceptably high, reaching levels of 95%, in spite of preceding promising data in pre-clinical studies (Hutchinson and Kirk, 2011). Although the background for this is multifactorial, novel *in vivo* models better capturing the relevant neoplastic niche, is clearly of great advantage. Pre-clinical studies on human tumors would, for increased clinical relevance, benefit from better capturing the neoplastic niche, i.e. permitting growth and progression to occur in the relevant in situ environment.

Importance of the microenvironment

As described earlier, currently used animal models, mainly xenografts and genetically modified animals, entail either the study of non-human cancer, or human cancer in a non-human environment, i.e. conditions that do not fully reflect the circumstances in the patient. Experimental environments that better mimic the natural milieu would be advantageous.

The ability of tumors to invade host tissues and metastasize is dependent on tumor-host interactions mediated by several molecular routes that normally enable important physiological functions, such as morphogenesis, neurogenesis, and angiogenesis for review see e.g. Geho et al. (Geho et al., 2005). Due to the cellular origin of the tumors studied in this project, markers related to neural crest migration are of particular interest here. Expression patterns of such molecules may be explored both as prognostic markers as well as possible therapeutic targets. Hence, current consensus supports the importance of a strong interplay with the surrounding tissue promoting tumor growth and spread. Many of the anti-angiogenesis drugs proposed in cancer therapy have been shown to play a central role in cell migration, firstly for the cells in the vessels, but possibly also for the tumor cells per se.

4.2 PAPER II

Aims of the study

As discussed above, animal models may not provide an appropriate system for studying human cancers and their treatment because of species differences. This paper concentrates on detecting advantages of a human cellular microenvironment, and thus species-specific interactions, in molecular studies of human tumor progression and invasiveness.

Tzukerman et al has previously described how experimental teratoma (see Paper I) can provide a general growth support for various human tumor cell lines (Tzukerman et al., 2003; Tzukerman et al., 2006). Here we take the teratoma model further from the original findings described by Tzukerman (Tzukerman et al., 2003; Tzukerman et al., 2006). We used a malignant melanoma cell line BL, inoculated into PSCT (in this publication referred to as the “hEST-model”) from two human embryonic stem cell lines, H9 and HS181 as well as mouse xenografts. Melanoma tumors were allowed to grow for two weeks and the growth was compared. BL melanoma cells were identified using XY chromosome specific FISH probe and the histology was compared by H&E staining. A wide panel of markers was used to compare the histopathological profile of the tumors growing in different model systems. A comparison was also done with the original patient tumor using the findings on the original sections from the patient archives.

The main findings were as follows:

Analogous histology of BL tumor in xenografts and PSCT model

Tumor histology showing high cellular density and low stroma contribution, from all the models was analogous, consisting of pleomorphic cells growing in a delimited densely packed manner. Melanoma cells showed highly malignant appearance with large nuclei, distinct nucleoli and a less prominent cytoplasm. Comparison from archival H&E-stained sections from the original tumor revealed that this cellular morphology was also similar to that of original BL patient.

Differences in cellular differentiation markers

Although showing similar histology, some differences in expression of melanoma specific markers were seen on tumors from different models. MelanA along with HMB45 and tyrosinase, are used as diagnostic markers for melanoma. BL melanoma growing as a xenograft showed high expression of HMB45, MelanA and Tyrosinase, while tumor cells in the PSCT were negative for these markers. Historical information from patient journal shows that the original tumor from the patient metastasis was diagnosed with only partly HMB45 staining and no MelanA expression. Hence xenograft model directed tumor cells to differentiate, while in PSCT the cells maintained a more undifferentiated state similar to the original patient metastasis and *in vitro* cultured cells.

PSCT-specific features of BL melanoma

Tropism

The BL tumor showed some special characteristics, while growing in the PSCT, which were not present in tumors growing as xenografts. A clear tropism was seen in the tumor integration process. For practical reasons, BL cells could not be directed to specific areas inside the teratoma and hence were injected in random positions in teratomas. Interestingly, BL showed an exclusive tropism for engraftment by almost always growing in areas compatible to condensed mesenchyme, analogous to neural crest development. Tumor cells were never found engrafted in areas other than condensed mesenchyme e.g., areas compatible with the primitive neuroectodermal development, secretory epithelial structures, cartilage etc. or in other organoid structures.

Human neovascularization in PSCT upon BL melanoma injections

Engraftment of BL tumor cells induced extensive neovascularization in the PSCT environment. BL cells were found surrounded by human vessels in the adjacent human stroma, indicated by positive staining for human CD31. As described previously by others and us the mature human vasculature in the teratoma is generally weak and the blood supply in teratoma is mainly from host (Gerecht-Nir et al., 2004; Gertow et al., 2004). Possibly, the integration of melanoma cells in the teratoma was initially

prioritized close to the mouse vessels, and the neo-angiogenesis in the surrounding stroma was a later event. Our findings are in line with the findings of Tzukerman et al who reported strong vascularization adjacent and within the tumor mass of A431 epidermoid carcinoma cell line growing in a similar teratoma model (Tzukerman et al., 2006). Contrary to Tzukerman et al, we found that the vessels within the tumor mass were of mouse origin.

Melanoma cells are known to induce vasculogenic mimicry, i.e. to support their own growth and progression by directing tumor cells to endothelial differentiation, which is usually indicated by the up-regulation of VE-cadherin (Carmeliet, 2003). This way tumor cells can participate in angiogenic process. Here, however the endothelial lining of human blood vessels was detected to be derived from hESC indicated by XX karyotype on FISH.

Additional more aggressive and metastatic BL phenotype in PSCT

The BL cells did not exhibit invasive behavior in xenografts, but did so in the PSCT model, as suggested by morphology supported also by the expression of S100A4 and Nodal. In PSCT exclusively, an additional, apparently more aggressive phenotype of BL cells was seen located at the borders of or infiltrating into the surrounding human loose mesenchymal fibrous stroma, forming an invasive front, which was positively identified by FISH analysis. These migratory cells had a desmoplastic, spindle-like appearance, expressing markers indicative of dedifferentiation and migration. Immunohistochemical findings of markers related to migrations and invasion was also in line with the phenotype of these cells, such as the S100A4, which is a marker of malignancy, was found exclusively in the invasive fronts in tumors growing in human teratoma model. The expression of S100A4, in xenograft was dispersed in single cells within the tumor mass. Nodal, another marker linked to dedifferentiation is known to be overexpressed in aggressive metastatic tumors including melanoma (Postovit et al., 2008; Topczewska et al., 2006), was only found dispersed in some tumor cells growing as xenograft but was focally exclusively expressed in the tumors cells lining the invasive front while growing in PSCT and not expressed in other parts of the tumors. Nodal is an embryonic morphogen belonging to the transforming growth factor beta superfamily (Schier, 2003; Shen, 2007). It plays a role in embryonic axes formation, induction of mesendoderm, patterning of nervous system and determining left and right asymmetry in vertebrates (Schier, 2003; Shen, 2007). Nodal has also been shown to maintain pluripotency of human embryonic stem cells (Vallier et al., 2004). Studies have shown that Nodal signalling reactivates in the neoplastic conditions (Topczewska et al., 2006). Several publications have presented the reappearance of Nodal in melanoma (Postovit et al., 2008; Topczewska et al., 2006), breast cancer (Postovit et al., 2008; Strizzi et al., 2008), gliomas (Lee et al., 2010), prostate cancer (Lawrence et al., 2011) and endometrial cancers (Papageorgiou et al., 2009). It is also evident that higher expression of nodal is specifically linked to more aggressive and invasive tumors like melanomas (Topczewska et al., 2006). Thus the expression of nodal in the BL melanoma cell line growing in PSCT, especially in the tumor cells migrating out and forming an invasive front while growing in the human embryonic environment is in line with the earlier findings on Nodal and it's link with aggressiveness. What makes it more interesting is the expression of markers liked to dedifferentiation and absence of

markers related to differentiation, which suggests a more aggressive phenotype of melanoma cells in the PSCT.

This study provided a proof of concept to study melanoma in the PSCT model. Since there are not many experimental *in vivo* melanoma models that are clinically relevant, the PSCT model could be potentially very useful tool to examine melanoma progression and it would also enable studies on species-specific tumor-host interactions.

4.4 PAPER III

Immature neural development in PSCT resembles the histology of neuroblastoma

Aims of the study
As described earlier, ectopic implantation of human pluripotent stem cells in mice results in differentiation defined as the formation of experimental teratoma. Notably, a prolonged immaturity of some neural components is regularly observed in benign PSCT rev by Cedervall et al. (Cedervall J, 2012). More specifically, immature neural areas in PSCT display histological resemblance with primitive neuroectodermal and neuroendocrine tumors. Similar histopathology, when appearing in patient samples for clinical diagnosis, is considered potentially malignant. However, in the PSCT model such histology is part of the chaotic but benign embryonic development (Paper I).

This raised a prospect that the embryonic microenvironment in PSCT is particularly well suited for studies on neural childhood tumors. In Paper II we demonstrated how PSCT could provide growth support for a melanoma cell line. In Paper III, we tested for the support of neuroblastoma of childhood origin, with the principle of recuperating a closer developmental match between the injected tumor and the microenvironment.

Here, we injected three human neuroblastoma (NB) cell lines (IMR-32, Kelly, and SK-N-BE(2)), into human pluripotent stem cells induced teratoma (PSCT) in SCID/beige mice and the tumor growth was evaluated for histopathology and marker profiles. Proliferative tumor cells could readily be identified by FISH analysis and immunohistochemistry, and uniquely distinguishable from other human cells in the PSCT. The comparative analysis of all tumor cell lines was done with the xenografts of respective line.

The main findings were as follows:

Tropism

All tumor injections resulted in the palpable tumor growth. All tumor lines showed a strict preference for integration, by always engrafting in areas compatible with undifferentiated embryonic loose mesenchymal stroma and never in areas compatible with other structures such as bone, muscles, gut, or other easily identifiable tissues in PSCT.

Noticeably, the undifferentiated stroma in the early embryo is formed by the embryonic loose mesenchyme. It is composed mainly of the mesodermal-derived cells, but includes also cells from other germ layers, e.g. ectodermal neural crest cells. It has a rich blood supply and includes also reticular fibers, creating a matrix. Cells of these embryonic connective tissues are typically spindle-, or star-shaped (stellate) and the amount of extracellular spaces between cells is relatively large. The extracellular matrix is filled with ground substance, which contains glycosaminoglycans, proteoglycans and glycoproteins. Similar appearance but with even more extracellular spaces and lesser reticular fibers is shown by mucoid connective tissue, which is a variant of embryonic connective tissue, present also in the umbilical cord (Wharton's jelly). Hence the embryonic loose mesenchyme in PSCT can be best understood as a kind of generalized connective tissue, which can consist of all connective tissue cell types.

In line with the above, the simplest explanation for the strong tropism of injected NB cells may be because the "open" nature of loose mesenchyme more easily giving space to new cells compared to other more dense coherent tissues. Alternatively, and more intriguing, NB tumor cells could also be attracted towards the environmental cues supplied by the embryonic loose mesenchyme and which could be of advantage for NB cells. The chemical composition of ground substance consisting of all known components in cell signaling processes, are potentially ideally situated for cues supporting NB cells. This hypothesis is also supported by our previous observations, where only minor kinetic time deviations were observed between the HS181 PSCT and human early neural development (paper 1). In this context, it is interesting that the PSCT developmental stage of neural development and the time point used here for injecting NB tumor cells (PSCT day 45) overlaps with the timing of when adrenal sympatical progenitors position themselves in embryonic mesenchyme in human neural crest development (E25-35; (Betters et al., 2010)). NB is assumed to derive from precursor cells of neural crest that specifically differentiate into the sympaticoadrenal lineage. Thus, these findings indicate that day 45-60 mature HS181 PSCT may provide an advantageous and timely embryonic human neuroectodermal niche for *in vivo* studies on pediatric neuroblastoma.

Expression of Nodal pathway

Further support for the hypothesis discussed above, comes from studies of the embryonic morphogen Nodal and its inhibitor Lefty (for background see Paper II) Prior to this study, it was reported that aggressive human melanoma re-express an aberrant and unregulated embryonic Nodal signaling pathway associated with tumor cell plasticity (Postovit et al., 2008; Topczewska et al., 2006). Therefore, in current study it was determined whether the embryonic morphogen Nodal (dependently or independently with its co-receptor Cripto-1) was expressed on NB tumors in PSCT and it was found that the NB cells here faithfully mirrored the Nodal/Cripto-1/Lefty expression of neural crest cells in mammals (Shen, 2007).
IMR-32 growing in PSCT or as xenorafts showed high expression of Nodal and Cripto but lefty was absent in PSCT and very low expression was shown in xenograft.

Histology of tumor cells

Tumor growth showed histological resemblance to that of clinical NB. In general, NB growth in both PSCT and as xenograft was undifferentiated or poorly differentiated having little or no interfoliating stroma or Schwann cell differentiation. Rosette formation, another trait of clinical NB, was commonly observed in tumors grafted in PSCT, but more rarely in the xenografts. Similar to clinical observations of NB, a considerably more heterogeneous growth pattern was found in the PSCT model compared to xenografts, as illustrated by organized and epitheloid-like growth in some areas and a dissociated and unstructured growth in other areas. A broader cellular diversity for tumors in PSCT was also suggested by the expression profiles, using markers for neurogenic (NSE), neuroendocrine (Synaptophysin and Chromogranin A), or melanocytic (Tyrosinase) phenotypes. Marker studies indicated differences between xenografts and PSCT, including tumor individual variations in phenotype.

Induction of neovascularization upon injection of NB cell in PSCT

Similar to the results in Paper II using a melanoma cell line, all NB tumors were well vascularized, with the blood vessels surrounding the tumor growth mainly of mouse origin but human origin of vessels was also detected by the positive expression of human CD31 cells. Detection of NB tumor nodules in close proximity of blood vessels was in line with the previously published results with various adult tumors (Paper II) and (Katz et al., 2009; Tzukerman et al., 2003; Tzukerman et al., 2006). In general the human vessels in PSCT were mainly located in the surrounding of the tumor growth. Interestingly, cells within SK-N-BE(2) xenograft tumors stained occasionally for human CD31, indicating a tumor cell differentiation into endothelial phenotype. In this way, a cell line-specific response to the microenvironment was observed. In general, SK-N-BE(2) tumors exhibited also the most aggressive phenotype. This NB line was originally derived from a bone marrow metastasis after several rounds of chemotherapy and radiation, and harbors a mutation in the p53 gene (ATCC).

It would be expected for this more aggressive cell line to be less dependent, and thus less responsive, to influences from the tumor surroundings. Accordingly, SK-N-BE(2) did not exhibit the same level of variation between the different model systems. In contrast to Kelly and IMR-32 NB cells it tended to show a sporadic differentiation in the xenografts, which was not seen in PSCT. The presence of human CD31 positive cells could be interpreted as SK-N-BE(2) tumor cells differentiating into an endothelial phenotype, a phenomenon denoted as “vascular mimicry”, previously described for tumors of various origins, including malignant melanoma (Maniotis et al., 1999), breast cancer (Postovit et al., 2008) and also NB (Pezzolo et al., 2007).

Influence from the embryonic environment, versus species differences

The differences observed in cellular diversity between the two models could be caused by either due to cues prompted from the embryonic environment vs. an “adult” environment or by species-specific cues induced by the PSCT cells i.e. human vs. mouse. To explore the influence of embryonic vs. species cues, the same tumors were also grown in the PSCT derived from mouse embryonic stem cells. The differences

observed in cellular diversity between xenografts and PSCT were influenced by both embryonic and species-specific cues. We found that the three NB cell lines varied in this respect. The IMR-32 line showed a similar growth pattern and histology in both the mouse and human PSCT models. Thus, growth pattern for IMR-32 correlated with the PSCT embryonic microenvironment and was not dependent on species. The Kelly and SK-N-BE(2) lines both showed a more differentiated tumor phenotype in the mouse PSCT as compared to the human PSCT indicating a species-specific influence on growth pattern.

This study described NB growth in PSCT and recapitulates clinical observations. We suggest the PSCT model to be an important and complement to xenograft analysis for NB, and thus facilitating clinical translation. Considering the extremely poor prognosis of malevolent NB, further development of new models is of utmost importance.

4.5 PAPER IV

Studies on clinical tumor specimens in PSCT

Aims of the study
The findings in paper II and III suggested that the microenvironment in PSCT provide adequate support for growth of neuroectodermal tumors. To test for clinical relevance of these findings, we next evaluated the PSCT for in situ growth and progression of ≤fresh or frozen/thawed tumor biopsy materials obtained from the surgery of childhood tumor patients at the Karolinska University Hospital.

Engraftment into PSCT

Tumor samples from the surgery of six children with; Supratentorial primitive neuroectodermal tumor (sPNET); Undifferentiated infratenturial tumor with sarcomatous features; Pilocytic astrocytoma of the brainstem; Classic medulloblastoma; Esthesio-NB and Neuroblastoma (NB), respectively, were transplanted into mature pluripotent stem cell-derived teratoma (PSCT). Each patient tumor specimen was transplanted into 2-6 PSCT and the tumors were allowed to engraft for 2-4 weeks. Engraftment was detected by XY chromosome specific FISH probes. H& E staining was used for morphological analysis and histopathological analysis was carried out using immunohistochemistry.

Three out of six tumors showed engraftment; sPNET, Esthesio-NB and NB. Esthesio-NB showed solitary cells or clusters of up to 14 cells, while NB and sPNET specimens gave rise to more protruding growth, reaching cohesive tumor areas. A detailed analysis was done on the latter two. Compared to the findings in paper III where all NB cell line give a palpable growth in both xenografts and PSCT systems, here it is a proof of the fact that primary tumor biopsies are harder to grow *in vivo*, likely due to their slow growth rate. There can be other explanations for the detection of only 50% tumor biopsies in PSCT. One limitation with the model was that tumor engraftments were limited to one month before the total size of the teratoma and tumor mass reached the maximum acceptable size, indicating that the model is more suited for faster growing tumors and some tumors analyzed in the study did not find time enough to engraft

considering their slower growth rate. Moreover, for technical reasons and time limitations we had to limit our search for tumor engraftment up to 100 sections, we can hope to find tumor engraftment if we cut the tissues deeper. This was possibly reflected in our findings with verified engraftment in three out of six cases, and cohesive growth was in fact only detected for the metastasizing NB and sPNET tumors. Notably, parallel xenograft-injections, of similar inoculation doses of tumor (approx. $1mm^3$), did not result in palpable tumor growth for any of the six tumors, after observations for up to four months. Therefore, selection of an appropriate and supportive model system is of crucial importance, not only for studying growth patterns but also for developing more effective anti-cancer therapies.

Tropism

A tissue tropism was observed for implanted clinical NB and sPNET specimens in PSCT, in line with our earlier findings on malignant melanoma cell line (paper II) and NB cell lines (paper III). Both NB and sPNET showed a specific tropism in that engraftment by NB cells were exclusively incorporating into areas of loose mesenchyme and the sPNET tumor was found in condensing neural ectoderm. Thus, sections from the PSCT model provided unique *in vivo* capturing of both progression and spread, using patient primary tumors. Neither tumor was detected in PSCT tissues identified as muscle, cartilage, gut, adrenal glands or epithelium of undefined origins. The finding on tissue tropism of the tumors studied in this thesis emphasizes the importance of establishing model systems that can better capture engraftment of neuroectodermal tumors compared to xenografts. This is also important to consider in pre-clinical testing of anti-cancer therapies where accurate prediction of efficacy in patients is essential. In oncology, the attrition rate in clinical trials following promising data in pre-clinical studies is disappointingly high, reaching levels of 80-95% (Hutchinson and Kirk, 2011). The background for this is multifactorial, however it would clearly be a benefit to have *in vivo* models better capturing the relevant neoplastic niche.
Thus, PSCT model studied in this thesis provides a promising complement to the existing pre-clinical models.

Vascularization

A perivascular preference of engraftment was evident since tumor cells were regularly observed in the immediate proximity of vessels. This is also in line with our previous findings on Melanoma and NB cell lines. The PSCT microenvironment is well supplied by mouse vessels and human vessels are generally sparse and immature (Cedervall J, 2012; Gerecht-Nir et al., 2004). However, others and we have observed enhanced vascularization in PSCT induced by the presence of tumor cells (Tzukerman et al., 2003; Tzukerman et al., 2006). Notably in the present study, both NB and sPNET were detected growing next to blood vessels, evidently taking advantage of a perivascular environment, previously suggested to influence tumorigenesis and/or aggressive behavior of neuroectodermal tumors (Axelson et al., 2005).

General histological findings

The tumor cell morphology was similar to that of the patient original tumor and matched also to that of the neighboring immature PSCT tissues for both NB and sPNET. The NB and sPNET tumors were both very undifferentiated and aggressive tumors, and these qualities were maintained after changing the environment to PSCT. Both tumors showed an expression profile, before and after transfer, of beta-Catenin+, CD117+, CD56+, Vimentin+, E-cad-, CD117+. A mesenchymal phenotype was presented for both the sPNET and NB, in the original patient tumor as well as when growing in PSCT, indicated by a robust staining of Vimentin and a lack of E-Cadherin.

However, while this profile was preserved, other changes in the molecular signature were indicated. The sPNET acquired the markers NSE (neurogenic) and Tyrosinase (melanocytic), indicating in this case a change to a more differentiated phenotype. The NB tumor on the other hand showed a loss of NFP and the neurogenic markers NSE, as well as a reduction or loss of the neuroendocrine markers CGA and Synaptophysin, suggesting a de-differentiation.

Also, the NB tumor showed loss of CD44 in the PSCT. CD44 is a cell surface glycoprotein involved in cell-cell interaction, cell adhesion and migration. It is known to have a high expression in the progression of colon, breast and non-hodgkin lymphoma (Goodison et al., 1999). However, Favrot et al have studied the role of CD44 in 52 NB patients and concluded that unlike other tumors, the absence of CD44 in NB indicated aggressive behavior (Favrot et al., 1993). Thus, the observed loss of also CD44 in PSCT could, together with the change of markers discussed above, have bearing on aggressiveness. Alternatively, in NB, cellular heterogeneity is a hallmark and NB is also known to vary in cytology, but not histology, possibly explaining some of the changes noted during growth in the PSCT (Favrot et al., 1986).

This study established for the first time that childhood neuroectodermal tumors could engraft in a PSCT environment, showing also tropism with striking similarities to the tumor conditions in the young patients. The influence of surrounding tissues for promoting tumor growth and spread is well recognized, and there is strong consensus that the ability to invade host tissues and metastasize is dependent and mediated by several molecular routes that normally enable important physiological functions, such as morphogenesis, neurogenesis, and angiogenesis (Geho et al., 2005).

In conclusion, the human PSCT microenvironment was found capable of supporting growth from patient childhood tumors of ectodermal origin, and providing striking similarities to the tumor conditions in the young patients.

6 CONCLUSIONS

Paper I

Studies of early and late events in experimental human teratoma, generated from the pluripotent stem cell line HS181, demonstrated the occurrence of an embryonic process including increasingly chaotic embryonic tissues. Further, an emerging organoid development was observed exhibiting cellular differentiation with close resemblance to that of the developing human embryo.

However, in line with earlier reports, a prolonged immaturity of some neural components was also observed. Similar histopathology when appearing in patient samples for clinical diagnosis is considered potentially malignant. Notably, in pluripotent stem cell induced teratoma (PSCT), such histology could be observed to be part of the chaotic but benign embryonic development.

Papers II and III

Based on the findings in Paper I, we observed predominant neural development in early PSCT and the histological resemblance of prolonged immature neural development in PCST to that of primitive neuroectodermal tumors, we hypothesized that the PSCT microenvironment could be well matched for studies of neuroectodermal tumors.

This concept was in Paper II confirmed using a melanoma cell line, and in Paper III using three neuroblastoma cell lines. Both experimental situations resulted in tumor growth and morphology with strong resemblance to that of clinical tumors.
Individual variations in molecular profiles for the three neuroblastoma cell lines were observed, providing additional information on the neuroblastoma tumor biology.

Paper IV

In this study we used, fresh or frozen, patient tumor materials and demonstrated for the first time that clinical childhood neuroectodermal tumors engraft into the PSCT environment. The patient tumor showed specific tissue tropism in their specific engraftment into developing mesenchymal and neuroectodermal tissues.

Sections from the PSCT model demonstrated **unique *in vivo* capturing of progression and micro invasion of the transplanted patient primary tumors** - with striking similarities to the tumor conditions in the young patient.

We conclude that the PSCT model delivers an *in vivo* environment allowing both NB and sPNET to maintain most of their original characteristics. This suggest that the PSCT environment is especially well suited for the assessment of childhood neuroectodermal tumors, and a strong complementary pre-clinical model for *in vivo* studies on childhood tumors.

7 FUTURE PROSPECTS

- More tumors should be tested to investigate the potential of the system to support different tumor types originating from different cell types. The embryonic potential should be used to study more embryonic tumors in the same system.

- Patient-specific iPSC giving rise to patient specific human environment will help understand the contribution of specific stroma to individual tumors.

- Next, this opens up for possibilities to gauge anti-cancer drug exposure and pharmacodynamics in this human *in vivo* environment, a well-needed complement to current pre-clinical models in anti-cancer drug development.

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9 REFERENCES

Albino, A.P., Nanus, D.M., Mentle, I.R., Cordon-Cardo, C., McNutt, N.S., Bressler, J., and Andreeff, M. (1989). Analysis of ras oncogenes in malignant melanoma and precursor lesions: correlation of point mutations with differentiation phenotype. Oncogene *4*, 1363-1374.

Albright, A.L., Wisoff, J.H., Zeltzer, P., Boyett, J., Rorke, L.B., Stanley, P., Geyer, J.R., and Milstein, J.M. (1995). Prognostic factors in children with supratentorial (nonpineal) primitive neuroectodermal tumors. A neurosurgical perspective from the Children's Cancer Group. Pediatric neurosurgery *22*, 1-7.

ATCC.

Attiyeh, E.F., London, W.B., Mosse, Y.P., Wang, Q., Winter, C., Khazi, D., McGrady, P.W., Seeger, R.C., Look, A.T., Shimada, H.*, et al.* (2005). Chromosome 1p and 11q deletions and outcome in neuroblastoma. The New England journal of medicine *353*, 2243-2253.

Axelson, H., Fredlund, E., Ovenberger, M., Landberg, G., and Pahlman, S. (2005). Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors. Seminars in cell & developmental biology *16*, 554-563.

Ball, N.J., Yohn, J.J., Morelli, J.G., Norris, D.A., Golitz, L.E., and Hoeffler, J.P. (1994). Ras mutations in human melanoma: a marker of malignant progression. The Journal of investigative dermatology *102*, 285-290.

Bastian, B.C. (2003). Understanding the progression of melanocytic neoplasia using genomic analysis: from fields to cancer. Oncogene *22*, 3081-3086.

Bauer, J., and Garbe, C. (2003). Acquired melanocytic nevi as risk factor for melanoma development. A comprehensive review of epidemiological data. Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society *16*, 297-306.

Beiske, K., Burchill, S.A., Cheung, I.Y., Hiyama, E., Seeger, R.C., Cohn, S.L., Pearson, A.D., and Matthay, K.K. (2009). Consensus criteria for sensitive detection of minimal neuroblastoma cells in bone marrow, blood and stem cell preparations by immunocytology and QRT-PCR: recommendations by the International Neuroblastoma Risk Group Task Force. British journal of cancer *100*, 1627-1637.

Betters, E., Liu, Y., Kjaeldgaard, A., Sundstrom, E., and Garcia-Castro, M.I. (2010). Analysis of early human neural crest development. Developmental biology *344*, 578-592.

Birch S, T.E. (1683). Philos Trans R Soc *13*, 281-284.

Blum, B., and Benvenisty, N. (2008). The tumorigenicity of human embryonic stem cells. Advances in cancer research *100*, 133-158.

Bown, N. (2001). Neuroblastoma tumour genetics: clinical and biological aspects. Journal of clinical pathology *54*, 897-910.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G.*, et al.* (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947-956.

Bray, F., Jemal, A., Grey, N., Ferlay, J., and Forman, D. (2012). Global cancer transitions according to the Human Development Index (2008-2030): a population-based study. The lancet oncology.

Brinster, R.L. (1974). The effect of cells transferred into the mouse blastocyst on subsequent development. The Journal of experimental medicine *140*, 1049-1056.

Brivanlou, A.H., Gage, F.H., Jaenisch, R., Jessell, T., Melton, D., and Rossant, J. (2003). Stem cells. Setting standards for human embryonic stem cells. Science *300*, 913-916.

Brodeur, G.M. (1998). Are bone marrow aspirates or biopsies the preferred method to detect metastatic neuroblastoma to bone marrow? Journal of pediatric hematology/oncology *20*, 297-298.

Brodeur, G.M. (2003). Neuroblastoma: biological insights into a clinical enigma. Nature reviews Cancer *3*, 203-216.

Brodeur, G.M., Pritchard, J., Berthold, F., Carlsen, N.L., Castel, V., Castelberry, R.P., De Bernardi, B., Evans, A.E., Favrot, M., Hedborg, F.*, et al.* (1993). Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *11*, 1466-1477.
Bronner-Fraser, M., and Stern, C. (1991). Effects of mesodermal tissues on avian neural crest cell migration. Developmental biology *143*, 213-217.
Caren, H., Kryh, H., Nethander, M., Sjoberg, R.M., Trager, C., Nilsson, S., Abrahamsson, J., Kogner, P., and Martinsson, T. (2010). High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset. Proceedings of the National Academy of Sciences of the United States of America *107*, 4323-4328.
Carmeliet, P. (2003). Angiogenesis in health and disease. Nature medicine *9*, 653-660.
Cedervall J, G.K., Damjanov I, Ährulnd-Richter L (2012). Characterization of human pluripotent stem cell-derived teratomas, in human stem cell manual: A laboratory guide. (Academin Press).
Claeson, M., Andersson, E.M., Wallin, M., Wastensson, G., Wennberg, A.M., Paoli, J., and Gonzalez, H. (2012). Incidence of cutaneous melanoma in Western Sweden, 1970-2007. Melanoma research *22*, 392-398.
Cohen, B.H., Zeltzer, P.M., Boyett, J.M., Geyer, J.R., Allen, J.C., Finlay, J.L., McGuire-Cullen, P., Milstein, J.M., Rorke, L.B., Stanley, P.*, et al.* (1995). Prognostic factors and treatment results for supratentorial primitive neuroectodermal tumors in children using radiation and chemotherapy: a Childrens Cancer Group randomized trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *13*, 1687-1696.
Cohn, S.L., Pearson, A.D., London, W.B., Monclair, T., Ambros, P.F., Brodeur, G.M., Faldum, A., Hero, B., Iehara, T., Machin, D.*, et al.* (2009). The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *27*, 289-297.
Cohn, S.L., and Tweddle, D.A. (2004). MYCN amplification remains prognostically strong 20 years after its "clinical debut". Eur J Cancer *40*, 2639-2642.
Cooke, M.J., Stojkovic, M., and Przyborski, S.A. (2006). Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. Stem cells and development *15*, 254-259.
Croy, B.A., and Chapeau, C. (1990). Evaluation of the pregnancy immunotrophism hypothesis by assessment of the reproductive performance of young adult mice of genotype scid/scid.bg/bg. Journal of reproduction and fertility *88*, 231-239.
Curtin, J.A., Busam, K., Pinkel, D., and Bastian, B.C. (2006). Somatic activation of KIT in distinct subtypes of melanoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *24*, 4340-4346.
Curtin, J.A., Fridlyand, J., Kageshita, T., Patel, H.N., Busam, K.J., Kutzner, H., Cho, K.H., Aiba, S., Brocker, E.B., LeBoit, P.E.*, et al.* (2005). Distinct sets of genetic alterations in melanoma. The New England journal of medicine *353*, 2135-2147.
Dahlback, H.S., Brandal, P., Gorunova, L., Widing, E., Meling, T.R., Krossnes, B.K., and Heim, S. (2011). Genomic aberrations in pediatric gliomas and embryonal tumors. Genes, chromosomes & cancer *50*, 788-799.
Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W.*, et al.* (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949-954.
De Both, N.J., Vermey, M., Groen, N., Dinjens, W.N., and Bosman, F.T. (1997). Clonal growth of colorectal-carcinoma cell lines transplanted to nude mice. International journal of cancer Journal international du cancer *72*, 1137-1141.
Dirks, P.B., Harris, L., Hoffman, H.J., Humphreys, R.P., Drake, J.M., and Rutka, J.T. (1996). Supratentorial primitive neuroectodermal tumors in children. Journal of neuro-oncology *29*, 75-84.
Edlundh-Rose, E., Egyhazi, S., Omholt, K., Mansson-Brahme, E., Platz, A., Hansson, J., and Lundeberg, J. (2006). NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. Melanoma research *16*, 471-478.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.
Favrot, M.C., Combaret, V., and Lasset, C. (1993). CD44--a new prognostic marker for neuroblastoma. The New England journal of medicine *329*, 1965.
Favrot, M.C., Frappaz, D., Maritaz, O., Philip, I., Fontaniere, B., Gentilhomme, O., Bailly, C., Zucker, J.M., Gentet, J.C., Kemshead, J.*, et al.* (1986). Histological, cytological and immunological analyses are complementary for the detection of neuroblastoma cells in bone marrow. British journal of cancer *54*, 637-641.
Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C., and Parkin, D.M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer Journal international du cancer *127*, 2893-2917.
Fernandez-Flores, A. (2012). Prognostic factors for melanoma progression and metastasis: from Hematoxylin-Eosin to genetics. Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie *53*, 449-459.
Fisher, J.P., and Tweddle, D.A. (2012). Neonatal neuroblastoma. Seminars in fetal & neonatal medicine *17*, 207-215.
Fuchs, E., and Segre, J.A. (2000). Stem cells: a new lease on life. Cell *100*, 143-155.
Gandini, S., Sera, F., Cattaruzza, M.S., Pasquini, P., Zanetti, R., Masini, C., Boyle, P., and Melchi, C.F. (2005). Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. Eur J Cancer *41*, 2040-2059.
Garbe, C., Buttner, P., Weiss, J., Soyer, H.P., Stocker, U., Kruger, S., Roser, M., Weckbecker, J., Panizzon, R., Bahmer, F.*, et al.* (1994a). Associated factors in the prevalence of more than 50 common melanocytic nevi, atypical melanocytic nevi, and actinic lentigines: multicenter case-control study of the Central Malignant Melanoma Registry of the German Dermatological Society. The Journal of investigative dermatology *102*, 700-705.
Garbe, C., Buttner, P., Weiss, J., Soyer, H.P., Stocker, U., Kruger, S., Roser, M., Weckbecker, J., Panizzon, R., Bahmer, F.*, et al.* (1994b). Risk factors for developing cutaneous melanoma and criteria for identifying persons at risk: multicenter case-control study of the Central Malignant Melanoma Registry of the German Dermatological Society. The Journal of investigative dermatology *102*, 695-699.
Garbe, C., Peris, K., Hauschild, A., Saiag, P., Middleton, M., Spatz, A., Grob, J.J., Malvehy, J., Newton-Bishop, J., Stratigos, A.*, et al.* (2012). Diagnosis and treatment of melanoma. European consensus-based interdisciplinary guideline - Update 2012. Eur J Cancer *48*, 2375-2390.
Geho, D.H., Bandle, R.W., Clair, T., and Liotta, L.A. (2005). Physiological mechanisms of tumor-cell invasion and migration. Physiology (Bethesda) *20*, 194-200.
Gepstein, L. (2002). Derivation and potential applications of human embryonic stem cells. Circulation research *91*, 866-876.
Gerecht-Nir, S., Osenberg, S., Nevo, O., Ziskind, A., Coleman, R., and Itskovitz-Eldor, J. (2004). Vascular development in early human embryos and in teratomas derived from human embryonic stem cells. Biology of reproduction *71*, 2029-2036.
Gerecht-Nir, S., Ziskind, A., Cohen, S., and Itskovitz-Eldor, J. (2003). Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. Laboratory investigation; a journal of technical methods and pathology *83*, 1811-1820.
Gertow, K., Przyborski, S., Loring, J.F., Auerbach, J.M., Epifano, O., Otonkoski, T., Damjanov, I., and Ahrlund-Richter, L. (2007). Isolation of human embryonic stem cell-derived teratomas for the assessment of pluripotency. Current protocols in stem cell biology *Chapter 1*, Unit1B 4.
Gertow, K., Wolbank, S., Rozell, B., Sugars, R., Andang, M., Parish, C.L., Imreh, M.P., Wendel, M., and Ahrlund-Richter, L. (2004). Organized development from human embryonic stem cells after injection into immunodeficient mice. Stem cells and development *13*, 421-435.
Godar, D.E. (2011). Worldwide increasing incidences of cutaneous malignant melanoma. Journal of skin cancer *2011*, 858425.
Goel, V.K., Lazar, A.J., Warneke, C.L., Redston, M.S., and Haluska, F.G. (2006). Examination of mutations in BRAF, NRAS, and PTEN in primary cutaneous melanoma. The Journal of investigative dermatology *126*, 154-160.

Goodison, S., Urquidi, V., and Tarin, D. (1999). CD44 cell adhesion molecules. Molecular pathology : MP *52*, 189-196.
Grimmer, M.R., and Weiss, W.A. (2006). Childhood tumors of the nervous system as disorders of normal development. Current opinion in pediatrics *18*, 634-638.
Grobe, N., Ruhle, H., and Eckelmann, G. (1990). [Hyperprolactinemia in malignant lymphomas]. Dtsch Med Wochenschr *115*, 1825-1827.
Gustafsson G , H.H., Å Vernby (2007). Childhood cancer incidence and survival in sweden 1984-2005. Report from the swedish childhood cancer registry. Swedish childhood cancer registry (Karolinska Institutet, Stockholm, Sweden).
Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.
Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.
Heath, J.A., Zacharoulis, S., and Kieran, M.W. (2012). Pediatric neuro-oncology: Current status and future directions. Asia-Pacific journal of clinical oncology *8*, 223-231.
Heins, N., Englund, M.C., Sjoblom, C., Dahl, U., Tonning, A., Bergh, C., Lindahl, A., Hanson, C., and Semb, H. (2004). Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells *22*, 367-376.
Hocker, T., and Tsao, H. (2007). Ultraviolet radiation and melanoma: a systematic review and analysis of reported sequence variants. Human mutation *28*, 578-588.
Hocker, T.L., Singh, M.K., and Tsao, H. (2008). Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside. The Journal of investigative dermatology *128*, 2575-2595.
Holly, E.A., Kelly, J.W., Shpall, S.N., and Chiu, S.H. (1987). Number of melanocytic nevi as a major risk factor for malignant melanoma. Journal of the American Academy of Dermatology *17*, 459-468.
Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A.M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., and Ahrlund-Richter, L. (2003). A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod *18*, 1404-1409.
Hutchinson, L., and Kirk, R. (2011). High drug attrition rates--where are we going wrong? Nature reviews Clinical oncology *8*, 189-190.
Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med *6*, 88-95.
Jakacki, R.I. (1999). Pineal and nonpineal supratentorial primitive neuroectodermal tumors. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery *15*, 586-591.
Janoueix-Lerosey, I., Lequin, D., Brugieres, L., Ribeiro, A., de Pontual, L., Combaret, V., Raynal, V., Puisieux, A., Schleiermacher, G., Pierron, G.*, et al.* (2008). Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature *455*, 967-970.
Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010). Cancer statistics, 2010. CA: a cancer journal for clinicians *60*, 277-300.
Johnsen, J.I., Kogner, P., Albihn, A., and Henriksson, M.A. (2009). Embryonal neural tumours and cell death. Apoptosis : an international journal on programmed cell death *14*, 424-438.
Johnson, J.I., Decker, S., Zaharevitz, D., Rubinstein, L.V., Venditti, J.M., Schepartz, S., Kalyandrug, S., Christian, M., Arbuck, S., Hollingshead, M.*, et al.* (2001). Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. British journal of cancer *84*, 1424-1431.
Joshi, V.V., Cantor, A.B., Altshuler, G., Larkin, E.W., Neill, J.S., Shuster, J.J., Holbrook, C.T., Hayes, F.A., and Castleberry, R.P. (1992). Recommendations for modification of terminology of neuroblastic tumors and prognostic significance of Shimada classification. A clinicopathologic study of 213 cases from the Pediatric Oncology Group. Cancer *69*, 2183-2196.
Katz, E., Skorecki, K., and Tzukerman, M. (2009). Niche-dependent tumorigenic capacity of malignant ovarian ascites-derived cancer cell subpopulations. Clinical

cancer research : an official journal of the American Association for Cancer Research *15*, 70-80.
Kerbel, R.S. (2003). Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived-but they can be improved. Cancer biology & therapy *2*, S134-139.
Keynes, R.J., and Stern, C.D. (1984). Segmentation in the vertebrate nervous system. Nature *310*, 786-789.
Klesse, L.J., and Bowers, D.C. (2010). Childhood medulloblastoma: current status of biology and treatment. CNS drugs *24*, 285-301.
Klimanskaya, I., Chung, Y., Meisner, L., Johnson, J., West, M.D., and Lanza, R. (2005). Human embryonic stem cells derived without feeder cells. Lancet *365*, 1636-1641.
Larsen, W.J. (2001). Human Embryology, Third edn (Churchull Livingstone).
Lawrence, M.G., Margaryan, N.V., Loessner, D., Collins, A., Kerr, K.M., Turner, M., Seftor, E.A., Stephens, C.R., Lai, J., Postovit, L.M*., et al.* (2011). Reactivation of embryonic nodal signaling is associated with tumor progression and promotes the growth of prostate cancer cells. The Prostate *71*, 1198-1209.
Lee, C.C., Jan, H.J., Lai, J.H., Ma, H.I., Hueng, D.Y., Lee, Y.C., Cheng, Y.Y., Liu, L.W., Wei, H.W., and Lee, H.M. (2010). Nodal promotes growth and invasion in human gliomas. Oncogene *29*, 3110-3123.
Lensch, M.W., and Ince, T.A. (2007). The terminology of teratocarcinomas and teratomas. Nature biotechnology *25*, 1211; author reply 1211-1212.
Leong, S.P., Gershenwald, J.E., Soong, S.J., Schadendorf, D., Tarhini, A.A., Agarwala, S., Hauschild, A., Soon, C.W., Daud, A., and Kashani-Sabet, M. (2011). Cutaneous melanoma: a model to study cancer metastasis. Journal of surgical oncology *103*, 538-549.
Leveine Arnold J., S.H.H. (1993). Molecular Genetics of Nervous System Tumors (United States of America: Wiley-liss).
Levenberg, S., Golub, J.S., Amit, M., Itskovitz-Eldor, J., and Langer, R. (2002). Endothelial cells derived from human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *99*, 4391-4396.
Levenberg, S., Huang, N.F., Lavik, E., Rogers, A.B., Itskovitz-Eldor, J., and Langer, R. (2003). Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. Proceedings of the National Academy of Sciences of the United States of America *100*, 12741-12746.
Look, A.T., Hayes, F.A., Nitschke, R., McWilliams, N.B., and Green, A.A. (1984). Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma. The New England journal of medicine *311*, 231-235.
Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger, P.C., Jouvet, A., Scheithauer, B.W., and Kleihues, P. (2007). The 2007 WHO classification of tumours of the central nervous system. Acta neuropathologica *114*, 97-109.
MacDougall, J.R., Croy, B.A., Chapeau, C., and Clark, D.A. (1990). Demonstration of a splenic cytotoxic effector cell in mice of genotype SCID/SCID.BG/BG. Cellular immunology *130*, 106-117.
Maio, M. (2012). Melanoma as a model tumour for immuno-oncology. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO *23 Suppl 8*, viii10-viii14.
Maniotis, A.J., Folberg, R., Hess, A., Seftor, E.A., Gardner, L.M., Pe'er, J., Trent, J.M., Meltzer, P.S., and Hendrix, M.J. (1999). Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. The American journal of pathology *155*, 739-752.
Maris, J.M. (2010). Recent advances in neuroblastoma. The New England journal of medicine *362*, 2202-2211.
Maris, J.M., Hogarty, M.D., Bagatell, R., and Cohn, S.L. (2007). Neuroblastoma. Lancet *369*, 2106-2120.
Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proceedings of the National Academy of Sciences of the United States of America *78*, 7634-7638.
Miller, S., Rogers, H.A., Lyon, P., Rand, V., Adamowicz-Brice, M., Clifford, S.C., Hayden, J.T., Dyer, S., Pfister, S., Korshunov, A*., et al.* (2011). Genome-wide

molecular characterization of central nervous system primitive neuroectodermal tumor and pineoblastoma. Neuro-oncology *13*, 866-879.
Monclair, T., Brodeur, G.M., Ambros, P.F., Brisse, H.J., Cecchetto, G., Holmes, K., Kaneko, M., London, W.B., Matthay, K.K., Nuchtern, J.G., *et al.* (2009). The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *27*, 298-303.
Mosse, Y.P., Laudenslager, M., Longo, L., Cole, K.A., Wood, A., Attiyeh, E.F., Laquaglia, M.J., Sennett, R., Lynch, J.E., Perri, P., *et al.* (2008). Identification of ALK as a major familial neuroblastoma predisposition gene. Nature *455*, 930-935.
Nagy, A., Gocza, E., Diaz, E.M., Prideaux, V.R., Ivanyi, E., Markkula, M., and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. Development *110*, 815-821.
Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *90*, 8424-8428.
Nakagawara, A., and Ohira, M. (2004). Comprehensive genomics linking between neural development and cancer: neuroblastoma as a model. Cancer letters *204*, 213-224.
Omholt, K., Platz, A., Kanter, L., Ringborg, U., and Hansson, J. (2003). NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. Clinical cancer research : an official journal of the American Association for Cancer Research *9*, 6483-6488.
Oosterhuis, J.W., and Looijenga, L.H. (2005). Testicular germ-cell tumours in a broader perspective. Nature reviews Cancer *5*, 210-222.
Palmieri, G., Casula, M., Sini, M.C., Ascierto, P.A., and Cossu, A. (2007). Issues affecting molecular staging in the management of patients with melanoma. Journal of cellular and molecular medicine *11*, 1052-1068.
Papageorgiou, I., Nicholls, P.K., Wang, F., Lackmann, M., Makanji, Y., Salamonsen, L.A., Robertson, D.M., and Harrison, C.A. (2009). Expression of nodal signalling components in cycling human endometrium and in endometrial cancer. Reproductive biology and endocrinology : RB&E *7*, 122.
Paulino, A.C., and Melian, E. (1999). Medulloblastoma and supratentorial primitive neuroectodermal tumors: an institutional experience. Cancer *86*, 142-148.
Pezzolo, A., Parodi, F., Corrias, M.V., Cinti, R., Gambini, C., and Pistoia, V. (2007). Tumor origin of endothelial cells in human neuroblastoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *25*, 376-383.
Pierce, G.B., Jr., Dixon, F.J., Jr., and Verney, E.L. (1960). Teratocarcinogenic and tissue-forming potentials of the cell types comprising neoplastic embryoid bodies. Laboratory investigation; a journal of technical methods and pathology *9*, 583-602.
Plaia, T.W., Josephson, R., Liu, Y., Zeng, X., Ording, C., Toumadje, A., Brimble, S.N., Sherrer, E.S., Uhl, E.W., Freed, W.J., *et al.* (2006). Characterization of a new NIH-registered variant human embryonic stem cell line, BG01V: a tool for human embryonic stem cell research. Stem Cells *24*, 531-546.
Platz, A., Ringborg, U., Brahme, E.M., and Lagerlof, B. (1994). Melanoma metastases from patients with hereditary cutaneous malignant melanoma contain a high frequency of N-ras activating mutations. Melanoma research *4*, 169-177.
Pollack, I.F. (2011). Multidisciplinary management of childhood brain tumors: a review of outcomes, recent advances, and challenges. Journal of neurosurgery Pediatrics *8*, 135-148.
Postovit, L.M., Margaryan, N.V., Seftor, E.A., and Hendrix, M.J. (2008). Role of nodal signaling and the microenvironment underlying melanoma plasticity. Pigment cell & melanoma research *21*, 348-357.
Quattrocelli, M., Thorrez, L., and Sampaolesi, M. (2012). Pluripotent stem cell derivation and differentiation toward cardiac muscle: novel techniques and advances in patent literature. Recent patents on drug delivery & formulation.
Reddy, A.T., Janss, A.J., Phillips, P.C., Weiss, H.L., and Packer, R.J. (2000). Outcome for children with supratentorial primitive neuroectodermal tumors treated with surgery, radiation, and chemotherapy. Cancer *88*, 2189-2193.

Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., and Ben-Hur, T. (2001). Neural progenitors from human embryonic stem cells. Nature biotechnology *19*, 1134-1140.
Salazar-Onfray, F., Lopez, M., Lundqvist, A., Aguirre, A., Escobar, A., Serrano, A., Korenblit, C., Petersson, M., Chhajlani, V., Larsson, O.*, et al.* (2002). Tissue distribution and differential expression of melanocortin 1 receptor, a malignant melanoma marker. British journal of cancer *87*, 414-422.
Samkari, A., Hwang, E., and Packer, R.J. (2012). Medulloblastoma/Primitive neuroectodermal tumor and germ cell tumors: the uncommon but potentially curable primary brain tumors. Hematology/oncology clinics of North America *26*, 881-895.
Sceats, E. In vitro tissue models: working in the third dimension.
Schier, A.F. (2003). Nodal signaling in vertebrate development. Annual review of cell and developmental biology *19*, 589-621.
Schöler, H.R. (2007). The potential of setm cells: An inventory (Ashgate Publishing).
Schor, N.F. (1999). Neuroblastoma as a neurobiological disease. Journal of neuro-oncology *41*, 159-166.
Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D.A., and Benvenisty, N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *97*, 11307-11312.
Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. (1983). Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature *305*, 245-248.
Seftor, E.A., Meltzer, P.S., Kirschmann, D.A., Margaryan, N.V., Seftor, R.E., and Hendrix, M.J. (2006). The epigenetic reprogramming of poorly aggressive melanoma cells by a metastatic microenvironment. Journal of cellular and molecular medicine *10*, 174-196.
Shen, M.M. (2007). Nodal signaling: developmental roles and regulation. Development *134*, 1023-1034.
Shimada, H., Ambros, I.M., Dehner, L.P., Hata, J., Joshi, V.V., and Roald, B. (1999a). Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer *86*, 349-363.
Shimada, H., Ambros, I.M., Dehner, L.P., Hata, J., Joshi, V.V., Roald, B., Stram, D.O., Gerbing, R.B., Lukens, J.N., Matthay, K.K.*, et al.* (1999b). The International Neuroblastoma Pathology Classification (the Shimada system). Cancer *86*, 364-372.
Shimada, H., Umehara, S., Monobe, Y., Hachitanda, Y., Nakagawa, A., Goto, S., Gerbing, R.B., Stram, D.O., Lukens, J.N., and Matthay, K.K. (2001). International neuroblastoma pathology classification for prognostic evaluation of patients with peripheral neuroblastic tumors: a report from the Children's Cancer Group. Cancer *92*, 2451-2461.
Snell, R.S. (2009). Clinical Neuroanatomy, Senventh edn (Lippincott Williams & Wikilns).
Staroselsky, A.N., Radinsky, R., Fidler, I.J., Pathak, S., Chernajovsky, Y., and Frost, P. (1992). The use of molecular genetic markers to demonstrate the effect of organ environment on clonal dominance in a human renal-cell carcinoma grown in nude mice. International journal of cancer Journal international du cancer *51*, 130-138.
Stevens, L.C., and Little, C.C. (1954). Spontaneous Testicular Teratomas in an Inbred Strain of Mice. Proceedings of the National Academy of Sciences of the United States of America *40*, 1080-1087.
Strizzi, L., Postovit, L.M., Margaryan, N.V., Seftor, E.A., Abbott, D.E., Seftor, R.E., Salomon, D.S., and Hendrix, M.J. (2008). Emerging roles of nodal and Cripto-1: from embryogenesis to breast cancer progression. Breast disease *29*, 91-103.
Takimoto, C.H. (2001). Why drugs fail: of mice and men revisited. Clinical cancer research : an official journal of the American Association for Cancer Research *7*, 229-230.
Teicher, B.A. (2006). Tumor models for efficacy determination. Molecular cancer therapeutics *5*, 2435-2443.

Teillet, M.A., Kalcheim, C., and Le Douarin, N.M. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. Developmental biology *120*, 329-347.
Terskikh, A.V., Bryant, P.J., and Schwartz, P.H. (2006). Mammalian stem cells. Pediatric research *59*, 13R-20R.
Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.
Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. Proceedings of the National Academy of Sciences of the United States of America *92*, 7844-7848.
Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., and Hearn, J.P. (1996). Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biology of reproduction *55*, 254-259.
Thomson, J.A., and Odorico, J.S. (2000). Human embryonic stem cell and embryonic germ cell lines. Trends in biotechnology *18*, 53-57.
Topczewska, J.M., Postovit, L.M., Margaryan, N.V., Sam, A., Hess, A.R., Wheaton, W.W., Nickoloff, B.J., Topczewski, J., and Hendrix, M.J. (2006). Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. Nature medicine *12*, 925-932.
Touboul, T., Hannan, N.R., Corbineau, S., Martinez, A., Martinet, C., Branchereau, S., Mainot, S., Strick-Marchand, H., Pedersen, R., Di Santo, J.*, et al.* (2010). Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology *51*, 1754-1765.
Träger, C. (2009). Neuroblastoma incidence, biological outcome with special emphasis on tyrosinase hydroxylase mrna in blood and bone marrow. (Karolinska Institutet, Stockholm, Sweden).
Tsao, H., Atkins, M.B., and Sober, A.J. (2004). Management of cutaneous melanoma. The New England journal of medicine *351*, 998-1012.
Tumilowicz, J.J., Nichols, W.W., Cholon, J.J., and Greene, A.E. (1970). Definition of a continuous human cell line derived from neuroblastoma. Cancer research *30*, 2110-2118.
Tzukerman, M., Rosenberg, T., Ravel, Y., Reiter, I., Coleman, R., and Skorecki, K. (2003). An experimental platform for studying growth and invasiveness of tumor cells within teratomas derived from human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *100*, 13507-13512.
Tzukerman, M., Rosenberg, T., Reiter, I., Ben-Eliezer, S., Denkberg, G., Coleman, R., Reiter, Y., and Skorecki, K. (2006). The influence of a human embryonic stem cell-derived microenvironment on targeting of human solid tumor xenografts. Cancer research *66*, 3792-3801.
Ulbright, T.M. (2005). Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc *18 Suppl 2*, S61-79.
Ulloa-Montoya, F., Verfaillie, C.M., and Hu, W.S. (2005). Culture systems for pluripotent stem cells. Journal of bioscience and bioengineering *100*, 12-27.
Vallier, L., Reynolds, D., and Pedersen, R.A. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Developmental biology *275*, 403-421.
Vallier, L., Touboul, T., Brown, S., Cho, C., Bilican, B., Alexander, M., Cedervall, J., Chandran, S., Ahrlund-Richter, L., Weber, A.*, et al.* (2009). Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. Stem Cells *27*, 2655-2666.
van Limpt, V., Schramm, A., van Lakeman, A., Sluis, P., Chan, A., van Noesel, M., Baas, F., Caron, H., Eggert, A., and Versteeg, R. (2004). The Phox2B homeobox gene is mutated in sporadic neuroblastomas. Oncogene *23*, 9280-9288.
van Noesel, M.M., and Versteeg, R. (2004). Pediatric neuroblastomas: genetic and epigenetic 'danse macabre'. Gene *325*, 1-15.
Virchow, R. (1863). Die Krankenhaften Geschwulste.

Wu, H., Goel, V., and Haluska, F.G. (2003). PTEN signaling pathways in melanoma. Oncogene *22*, 3113-3122.
Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nature biotechnology *19*, 971-974.